

MONOGRAPHS ON BIOCHEMISTRY

EDITED BY

R. H. ADERS PLIMMER, D.Sc.

AND

F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S

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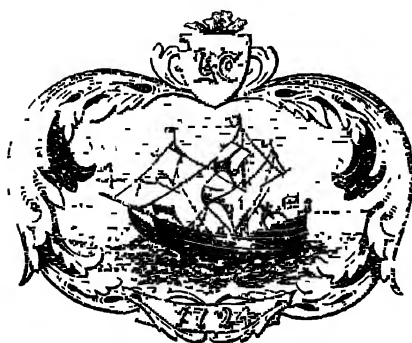
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THE
CHEMICAL CONSTITUTION
OF
THE PROTEINS

BY
R. H. ADERS PLIMMER, D.Sc.
ASSISTANT PROFESSOR OF PHYSIOLOGICAL CHEMISTRY IN, AND FELLOW OF
UNIVERSITY COLLEGE, LONDON

IN TWO PARTS
PART I



LONGMANS, GREEN, AND CO.
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1908

Dedicated
TO
EMIL FISCHER

THE MASTER OF
ORGANIC CHEMISTRY IN ITS RELATION TO BIOLOGY

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield

full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

P R E F A C E.

THE substance Protein, which constitutes the most important part of the material basis of all animal and vegetable life, has naturally attracted the attention and energy of numerous investigators throughout the past century. Progress in the study of this subject, on account of its difficulty, has been exceedingly slow, and it is only of recent years that the discovery of new methods by Emil Fischer has enabled us to increase our knowledge to its present extent. By these methods we have been able to advance from the conception of "albumin" to its actual separation into numerous units, and also to determine their arrangement in the molecule. On this account a monograph embodying the results of the most recent investigations, together with their connections with the work of the other and earlier investigators, needs no excuse for its appearance, as the subject is now being studied in every direction.

On account of the mass of material connected with the subject, this monograph has exceeded the proposed limit in length, and consequently it has become necessary to divide it into two parts:—

- I. { The Chemical Composition of the Protein Molecule.
The Chemical Constitution of its Units.
- II. The Synthesis of the Proteins.

R. H. A. P.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS.

PART I.

INTRODUCTION.

THE proteins, of which we know some forty or fifty natural ones occurring in both animals and plants, are divided, according to their origin, solubility, coagulability on heating and other physical characteristics, into the following groups:—

- I. Protamines, *e.g.*, salmine, sturine, clupeine, scombrine, cyclopteryne, cyprinine
- II. Histones, *e.g.*, thymus histone, Lota histone, Gadus histone, histone from blood corpuscles.
- III. Albumins, *e.g.*, ovalbumin, conalbumin, serum albumin, various plant albumins.
- IV Globulins, *e.g.*, serum globulin, fibrinogen and its derivative fibrin, myosinogen and its derivative myosin. Crystalline vegetable globulins: edestin, excelsin
- V. Glutelins, *e.g.*, legumin, conglutin, amandin, occurring in plants, soluble in very dilute alkali.
- VI. Gliadins, *e.g.*, wheat-gliadin, hordein, zein, occurring in cereals, soluble in 70-80 per cent. alcohol.
- VII. Phosphoproteins, *e.g.*, caseinogen, vitellin, ichthulin.
- VIII. Scleroproteins, *e.g.*, keratin from hair, horn, feathers, egg-membrane. Collagen, gelatin, elastin. Silk-fibroin, silk-gelatin.
- IX. Conjugated Proteins :—
 - (a) Nucleoproteins: nucleic acid in combination with protein, generally I., II., III.
 - (b) Chromoproteins: chromogenic substance in combination with protein, *e.g.*, hæmoglobin.
 - (c) Glucoproteins: carbohydrate in combination with protein, *e.g.*, mucin, ovomucoid.

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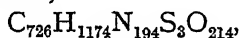
X. Derivatives of Proteins:—

- (a) Metaproteins, *e.g.*, acid-albumin, alkali-globulin.
- (b) Proteoses, *e.g.*, caseose, albumose, globulose.
- (c) Peptones, *e.g.*, fibrinpeptone.
- (d) Polypeptides, *e.g.*, glycyl-alanine, leucyl-glutamic acid, a tetrapeptide (2 glycine+1 alanine+1 tyrosine).

Except the protamines, the histones and the derivatives of the proteins, all the proteins contain carbon, hydrogen, nitrogen, sulphur and oxygen, and they possess the following elementary composition:—

C	51-55	per cent.
H	7	„
N	15-17	„
S	0.4-2.5	„
O	20-30	„

from which a formula such as,



which is that of globin, the basis of hæmoglobin, can be calculated.

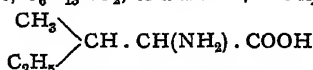
The phosphoproteins and the nucleoproteins contain also the element phosphorus; in the former, probably combined directly with one of the constituents of the protein molecule, in the latter, combined with a purine base or a carbohydrate, which substances constitute nucleic acid.

Investigations upon their chemical constitution have been carried on now for nearly a century, but it is only during the last ten years that, by the work of Emil Fischer and his pupils, any clear view has really been obtained of their actual constitution. The main result of these investigations is that the protein molecule is built up of a series of amino acids, which form the basis of their composition, and of which the following have been definitely determined:—

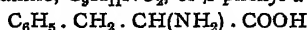
A. Mono-aminomonocarboxylic acids.

1. Glycine, $C_2H_5NO_2$, or amino-acetic acid.
 $CH_2 \cdot (NH_2) \cdot COOH$
2. Alanine, $C_3H_7NO_2$, or α -aminopropionic acid.
 $CH_3 \cdot CH(NH_2) \cdot COOH$
3. Valine, $C_5H_{11}NO_2$, or α -aminoisovaleric acid.
 $\begin{array}{l} CH_3 \\ \quad \diagup \\ CH_2 \end{array} \rangle CH \cdot CH(NH_2) \cdot COOH$
4. Leucine, $C_6H_{13}NO_2$, or α -aminoisocaproic acid.
 $\begin{array}{l} CH_3 \\ \quad \diagup \\ CH_2 \end{array} \rangle CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

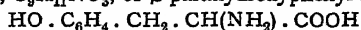
5. Isoleucine, $C_6H_{13}NO_2$, or α -amino- β -methyl- β -ethyl-propionic acid.



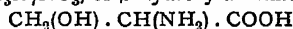
6. Phenylalanine, $C_9H_{11}NO_2$, or β -phenyl- α -aminopropionic acid.



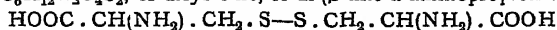
7. Tyrosine, $C_9H_{11}NO_3$, or β -parahydroxyphenyl- α -aminopropionic acid



8. Serine, $C_3H_7NO_3$, or β -hydroxy- α -aminopropionic acid.

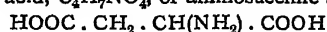


9. Cystine, $C_6H_{12}N_2O_4S_2$, or dicysteine, or di-(β -thio- α -aminopropionic acid).

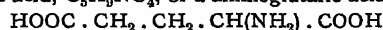


B. Monoaminodicarboxylic acids.

10. Aspartic acid, $C_4H_7NO_4$, or aminosuccinic acid.

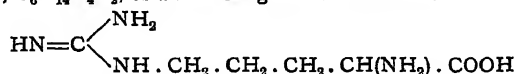


11. Glutamic acid, $C_5H_9NO_4$, or α -aminoglutaric acid.



C. Diaminomonomocarboxylic acids.

12. Arginine, $C_6H_{14}N_4O_2$, or α -amino- δ -guanidinovalerianic acid.

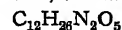


13. Lysine, $C_6H_{14}N_2O_2$, or α , ϵ -diaminocaproic acid.



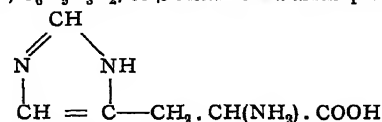
D. Diamino-oxy-monocarboxylic acid.

14. Caseinic acid, or diamino- γ -oxydodecanic acid

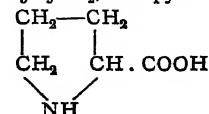


E. Heterocyclic compounds.

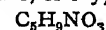
15. Histidine, $C_6H_9N_3O_2$, or β -imidazole- α -aminopropionic acid.



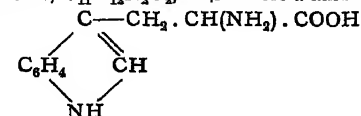
16. Proline, $C_5H_9NO_2$, or α -pyrrolidine carboxylic acid



17. Oxypyrrolidine, or oxypyrrolidine carboxylic acid.



18. Tryptophane, $C_{11}H_{12}N_2O_2$, or β -indole- α -aminopropionic acid.



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This long list is sufficient evidence of the complexity of the protein molecule; and, as yet, it seems to be still incomplete, for several other products have been described. They are not here included, since their presence in the molecule still requires confirmation.

These amino acids are known as the foundation-stones or units of the protein molecule. Before the constitution of a protein can be determined, it is essential that both the amount of any of these units and their chemical composition be known with certainty. The combination together of these amino acids has then to be determined. Consequently the study of the chemical constitution of the protein molecule must be divided into three main sections:—

- I. The Chemical Composition of the Protein Molecule.
- II. The Chemical Constitution of its Units.
- III. The Synthesis of the Proteins.

SECTION I

THE CHEMICAL COMPOSITION OF THE PROTEIN MOLECULE.

THE methods which have been employed for the purpose of determining the composition of the protein molecule have been many and various. They may be classified under four headings:—

- (1) Fusion with alkali.
- (2) Oxidation with permanganate, chromic acid, etc.
- (3) Action of halogens.
- (4) Hydrolysis.

Of these, the last, that of hydrolysis, has thrown most light on the darkness of this complex problem. Hydrolysis has been effected by the action of acids, of alkalies and of the various proteoclastic enzymes which occur in plants and animals, and is practically the only method by which we have attained to our present knowledge. Proteins were first hydrolysed by acids in 1820 by Braconnot, who used dilute sulphuric acid; between 1850 and 1875 hydrochloric acid was most frequently employed as the hydrolysing agent by Ritthausen, Hlasiwetz and Habermann and others, and from 1870 to 1880 Schutzenberger employed baryta water under pressure. The action of vegetable enzymes on proteins has been studied chiefly by Schulze and his co-workers, that of animal enzymes by Kuhne, Kossel, Kutscher, Drechsel and numerous other investigators.

As the result of hydrolysis a complex mixture of all, or nearly all, the previously mentioned units is obtained. These have been isolated by various methods based upon the fractional crystallisation of the compounds themselves, or of their copper, silver and other salts. Only when one or more of the amino acids occurred in somewhat large amounts was their isolation and characterisation effected; their amount seldom reached a value higher than 20 per cent. of the total quantity and the remainder was represented by uncrystallisable syrups of unknown nature. A great advance was made when Drechsel discovered that the protein molecule contained diamino acids as well as monoamino acids, and to Kossel and Kutscher we owe our chief knowledge concerning their isolation and estimation. Emil Fischer, in 1901, by his

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study of the amino acids and their derivatives, introduced a new method of isolating and separating the monoamino acids, which depended upon the fractional distillation *in vacuo* of their esters, and which is now commonly known as the *ester method*. This method, though not yet really quantitative, has enabled us to obtain a knowledge of some 70 per cent. of the total products resulting by hydrolysis, and it has shown us that phenylalanine, serine and alanine, which were only known to occur in a few, were present in all proteins, and that phenylalanine in its distribution was the principal aromatic constituent, for it often exceeds in amount that of tyrosine and occurs when this latter is absent. Further, it has demonstrated the presence of two new compounds, proline and oxyproline. In their isolation and estimation the various units are therefore divided into two main groups:—

- I. The Monoamino acids, including proline and oxyproline
- II. The Diamino acids, including histidine.

I. THE MONOAMINO ACIDS.

The Isolation and Estimation of Tyrosine, Cystine and Diaminotrioxydodecanic Acid.

Three of the amino acids, namely, tyrosine, cystine and diaminotrioxydodecanic acid are characterised by their extremely slight solubility in neutral aqueous solutions. They are therefore easily obtained after hydrolysis by acids by neutralising and concentrating the solution, when they crystallise out.

The separation of cystine and tyrosine as they are obtained by hydrolysis with hydrochloric acid was described by Morner in 1901. The protein—hair, keratin from horn, eggshells, etc.—was boiled with five times its quantity of 13 per cent hydrochloric acid under a reflux condenser on a water bath for six to seven days. The solution was then decolorised with charcoal and evaporated *in vacuo*, and the residue dissolved in 60 to 70 per cent. alcohol. The two acids then crystallised out on neutralising with soda, and were separated by fractional crystallisation from ammonia; if much tyrosine was present it separated out first, but if cystine exceeded tyrosine in quantity this compound crystallised out first; the remainder was only separated with difficulty. Embden separated the mixture of the two acids by means of very dilute nitric acid, in which tyrosine is very easily soluble, but cystine with difficulty. Their separation may also be effected by precipitation with mercuric sulphate in 5 per cent. sulphuric acid solution in which the mercury compound of tyrosine is soluble (Hopkins and Cole).

Hydrolysis by sulphuric acid possesses one great advantage over that by hydrochloric acid, as it can be subsequently completely removed by baryta. This method was employed by Fischer for obtaining tyrosine and diaminotrioxydodecanic acid from proteins, such as caseinogen, which contains very little cystine. The protein was hydrolysed by boiling with five to six times its quantity of 25 per cent. sulphuric acid for twelve to fifteen hours; the solution, after filtration, was diluted with twice its volume of water and neutralised with barium carbonate, or a strong solution of baryta, the excess of which was then removed by dilute sulphuric acid. The solution, together with the water used in thoroughly washing out the precipitate of barium sulphate, was then evaporated down, until these acids crystallised out. They were separated from one another by phosphotungstic acid, which precipitated the diaminotrioxydodecanic acid, and they were estimated by weighing, after removal of the phosphotungstic acid by baryta.

On account of the insolubility of these compounds and the difficulty of filtering and completely washing out the barium sulphate precipitate, in order to abstract from it the whole of the tyrosine, Abderhalden and Teruuchi, in the case of silk, have hydrolysed the protein with hydrochloric acid, the greater part of which was then removed by evaporation *in vacuo*; the remainder of the hydrochloric acid was then estimated in a small aliquot portion, and then separated quantitatively by neutralising with the calculated amount of caustic soda. The tyrosine then crystallised out, and was purified by recrystallisation from water.

A new method of determining the presence of tyrosine by bromination was introduced by Horace Brown and employed by Adrian Brown and Millar in 1906 for estimating the rate at which tyrosine is split off from proteins by the action of trypsin. This method might be used for the estimation of tyrosine in proteins; its non-employment may be due to the fact that tryptophane and also histidine react with bromine and might thus vitiate the result for tyrosine.

The Isolation and Estimation of the other Monoamino Acids.

For the preparation and estimation of the monoamino acids, hydrolysis by hydrochloric acid is more convenient than that by sulphuric acid. It was formerly carried out in the presence of stannous chloride (Hlasiwetz and Habermann) in order that the solution should remain colourless, instead of becoming dark brown, but this addition is not essential, as was shown by Cohn, and was not used by E. Fischer in his researches. Hydrolysis by hydrochloric acid is carried out by heating the protein with three times its quantity of concentrated hydrochloric acid

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of specific gravity 1.19, when it gradually passes into solution, if the flask in which it is contained be occasionally shaken and slightly warmed, and then boiling under a reflux condenser for five to ten hours, depending on the particular protein, and until the biuret reaction has completely disappeared. The solution, which may become at first violet, becomes finally of a dark brown colour and a portion of the hydrochloric acid is evolved as gas; it is boiled with charcoal, filtered from humin substances (secondary products probably arising from carbohydrate) and fatty material and consists of a solution of amino acids in 25 per cent. hydrochloric acid.

It is concentrated *in vacuo* to a small volume, and glutamic acid, if present in any large amount, is removed as its hydrochloride by saturating the solution with dry gaseous hydrochloric acid and allowing to stand at 0° C. for some days, when glutamic acid hydrochloride crystallises out. This occurs in the case of caseinogen and certain vegetable proteins, which contain from 10 to 40 per cent. of this amino acid. The glutamic acid hydrochloride is filtered off after adding an equal volume of ice-cold alcohol, redissolved in water, boiled with charcoal and again precipitated as hydrochloride by saturating the solution with gaseous hydrogen chloride, and weighed.

The solution thus freed from the greater part of the glutamic acid is again concentrated *in vacuo* at a low temperature to a thick syrup; this is dissolved in absolute alcohol (3 litres to 1 kilo. protein) and the amino acids are esterified by saturating the alcohol with dry gaseous hydrochloric acid at the ordinary temperature and then warming on the water bath for half an hour. In the process of esterification a large amount of water is formed, which prevents complete esterification; the alcohol is therefore evaporated off *in vacuo* at a temperature below 50° C., and the resulting syrup again dissolved in absolute alcohol and saturated with dry gaseous hydrochloric acid. In some cases it may be necessary to repeat this operation once more. At this stage, glycine, if it occurs in the protein, *e.g.*, in gelatin, in any considerable amount, is separated as glycine ester hydrochloride by seeding the solution with a crystal of this compound and allowing to stand for twenty-four hours at 0° C. It is filtered off while still cold, and the mother-liquor, on further concentration and saturation again with hydrochloric acid, may give another crop of glycine ester hydrochloride, so that almost the whole of the glycine may be isolated in this way. One recrystallisation from alcohol suffices to purify it and it is characterised by its melting point of 144° C. and analysis.

The filtrate containing the esters of the hydrochlorides of the other

amino acids is then concentrated to a syrup *in vacuo* at 40° C. and from this syrup the free esters are extracted as follows

About an equal volume of water is added to dissolve the syrup, and, if 1 kilo. of protein has been used, it is divided into four or six portions for convenience and to ensure the subsequent thorough cooling; to each portion one to two volumes of ether are added, and the mixture is thoroughly cooled in a freezing mixture of ice and salt; strong caustic soda is now added till the free hydrochloric acid is neutralised, and then a considerable excess of finely granulated potassium carbonate. The feebly basic esters of aspartic and glutamic acids, which are very sensitive to free alkali, are thus liberated and are dissolved by the ether, which is quickly poured off and replenished by a fresh quantity. The addition of 33 per cent. caustic soda in small portions at a time and of solid potassium carbonate sets free the other esters from their hydrochlorides; these are dissolved by the ether, which is frequently renewed throughout the process and thoroughly mixed with the mass of salt and potassium carbonate; sufficient caustic soda must be added to combine with the whole of the hydrochloric acid, and as much potassium carbonate as is necessary to form a pasty mass in order that the free esters, which are very easily soluble in water, are salted out and dissolved by the ether. A large amount of ether is required for this extraction, and an essential condition is that, throughout the process of extraction, the various portions be kept thoroughly cold by shaking in the freezing mixture.

The several ethereal extracts are then dried by shaking for about fifteen minutes with potassium carbonate, they are then combined together and allowed to stand for twelve hours with anhydrous sodium sulphate.

The ether is next evaporated off, preferably *in vacuo* at the ordinary temperature, as in this way the lower boiling esters do not distil with the ether and the danger of decomposing them by a higher temperature is avoided, and a brown oil, consisting of the esters of the amino acids, is obtained, which is fractionally distilled *in vacuo*.

By this method of extracting the esters from their hydrochlorides, neither that of tyrosine, which remains behind combined with alkali, nor those of the diamino acids, which are soluble with difficulty in ether, are obtained. This is advantageous for the subsequent process of separation, but the method has the disadvantage that the whole quantity of esters is not taken up by the ether on account of their destruction by the alkali. In order to avoid their loss, the mass of carbonate is treated with excess of hydrochloric acid and evaporated down, the potassium

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chloride being filtered off as it separates out; the residue is extracted with alcohol and the above process of esterification is repeated.

In order to avoid this loss and to obtain the amino acids as completely as possible, another method was introduced to liberate the esters from their hydrochlorides, *i.e.*, by treatment with sodium ethylate. The hydrochloric acid is therefore removed as completely as possible by evaporation *in vacuo* and the mixture of ester hydrochlorides is dissolved in five times its quantity of absolute alcohol. The amount of chlorine is estimated in a small portion of this, and to the remainder the calculated quantity of sodium dissolved in absolute alcohol, so as to make a 3 per cent. solution, is added. The sodium chloride formed is filtered off, and the alcohol is removed by evaporation *in vacuo*. A small quantity of the lower boiling esters of the amino acids passes into the distillate with the alcohol, but is recovered by acidifying with hydrochloric acid and evaporating when the amino acid hydrochlorides are obtained. A dark brown oil again results, which is fractionally distilled *in vacuo*. Although this method prevents loss by the action of alkali, the yield of the higher boiling fractions is not so great on account of the more complex nature of the mixture of esters. The residue which does not distil contains the tyrosine, the diamino acids and other substances.

The fractional distillation of the brown oil, which is obtained by either of these methods, is carried out firstly at a pressure of 10 to 12 mm. produced by a water pump, and then at a pressure of 0.5 mm. produced by a Geryck vacuum pump, as described by Fischer and Harries.¹

The temperatures at which the various fractions are collected are those of the vapours of the esters at 10 mm. pressure, and those of the water bath at 100° C. and of an oil bath, which replaces the water bath for the higher temperatures up to 160° C., at 0.5 mm. pressure.

The lower boiling fractions are again distilled *in vacuo* to obtain a further fractionation, but each fraction, even then, does not generally contain a single ester of an amino acid; in the case of the higher boiling fractions a second fractionation is not essential, since the esters contained in them can be separated by their varying solubility in water, ether and petroleum ether. The following table shows the fractions which are collected, and the amino acid esters which they may contain —

¹ In this process liquid air is used for condensing the vapours in order to preserve the high vacuum; carbonic acid has been used by other investigators, and Levene and van Slyke have recently employed sulphuric acid, cooled by a freezing mixture, as an absorbent for this purpose.

Temperature.	Pressure.	Esters of
Fraction I. To 40° (vapour) .	10 mm.	Glycine, alanine.
„ II. 40-60° (vapour) .	10 mm.	Alanine, leucine, proline.
„ III. 60-90° (vapour) .	10 mm.	Valine, leucine, proline.
„ IV. 100° (water bath) .	0.5 mm.	Leucine, proline.
„ V. 100-130° (oil bath) .	0.5 mm.	Phenylalanine, aspartic acid, glutamic acid, serine.
„ VI. 130-160° (oil bath) .	0.5 mm.	Phenylalanine, glutamic acid, aspartic acid, serine.

The next operation consists in the reconversion of the esters into the amino acids. In the case of the lower boiling fractions, this reconversion is effected by boiling the esters with five to six volumes of water under a reflux condenser for six to seven hours, until the alkaline reaction has disappeared; in the case of the higher boiling fractions, which contain the esters of aspartic and glutamic acids, the reconversion is effected by boiling with baryta water for one and a half to two hours; hydrolysis by water alone only converts these esters into their acid esters. Phenylalanine ester is converted into its hydrochloride by evaporation to dryness with hydrochloric acid.

The Separation and Characterisation of the Individual Monoamino Acids.

The separation, estimation and characterisation of each constituent contained in an ester fraction has still to be carried out after the conversion into the free amino acid, and for each amino acid a somewhat special process has to be performed, which is best described for each individual product.

Glycine.—As previously stated, if glycine occurs in large amounts in a protein, the greater quantity of it is separated as ester hydrochloride before the esters are liberated from their hydrochlorides and fractionally distilled. The remainder is contained in the first ester fraction, from which it is obtained by again esterifying and separating as ester hydrochloride. It is identified by its melting point and by elementary analysis, and its amount determined by its yield.

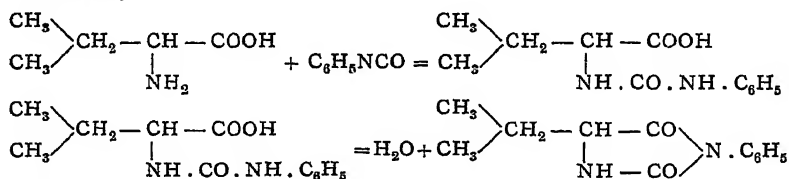
Alanine.—After the glycine has been removed as above described, the alanine is obtained by crystallisation after removal of the hydrochloric acid by boiling with lead hydroxide.

When mixed with valine, leucine and proline, the two former separate in the first fractions on crystallisation when their solution is evaporated; if present in large amounts it crystallises out in an almost pure state. It is separated from proline by evaporating to dryness and boiling with

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five to six times the quantity of absolute alcohol, which dissolves the proline, leaving the alanine, which is then purified by crystallisation from water. It may be characterised by its melting point, its rotation in hydrochloric acid solution, by elementary analysis, or by conversion into its benzoyl derivative, which may, however, have a rather low melting point on account of its partial racemisation. It is estimated by its yield.

Valine.—This amino acid is contained mixed with leucine in the fractions of the esters which boil between 60° and 90° C. Its isolation and separation from leucine is of extreme difficulty, since these compounds, as well as their copper salts into which they are converted by boiling with freshly precipitated cupric oxide, tend to form mixed crystals. Its isolation was only effected by these means in certain cases, and its amount is really much more than the figures represent from its yield. It is best characterised by conversion into its phenylhydantoine derivative by treatment with phenylisocyanate in alkaline solution. The phenylureido acid is first formed, and this loses a molecule of water, as shown by Mouneyrat, and is changed into its anhydride or phenylhydantoine by treatment with hydrochloric acid. The following reactions occur:—



Leucine.—The greater part of the leucine is contained in the ester fractions, which boil between 70° and 90° C. It generally occurs in considerable amounts in the protein, and is obtained by crystallisation from water, in which it is less soluble than the other amino acids which may be present. It is seldom present in its pure, optically active form, as this is easily racemised, and the various crops of crystals most probably also contain isoleucine. It is more easily isolated by completely racemising the mixture of amino acids contained in this fraction by heating in an autoclave with baryta to 160–180° C., and then, after removal of the baryta, separating it by crystallisation. The difficulty of separating it from the other amino acids, especially valine and isoleucine, makes an exact quantitative estimation almost impossible. The values which have been found are therefore minimal ones, and they will also include in many cases the yield of isoleucine.

Isoleucine.—The separation of leucine and isoleucine has been

carried out by F. Ehrlich, who makes use of the different solubility of the copper salts of these two amino acids in methyl alcohol. Levene has also employed this method.

Proline.—This is the only product of hydrolysis obtained from an ester fraction which is soluble in alcohol; it is also much more easily soluble in water than the other products with which it is present and therefore is somewhat easily separated, as it remains in the mother-liquor after these have crystallised out. The solution, in which it is contained, is evaporated to dryness and extracted with absolute alcohol; the combined alcoholic extracts from the several fractions are evaporated to dryness and taken up by absolute alcohol several times, so as to remove small amounts of the other amino acids, which, though insoluble in alcohol, are dissolved when proline is present.

As thus obtained, the proline is a mixture of the optically active and the racemic forms; these are separated by conversion into their copper salts and treatment with absolute alcohol which dissolves that of the optically active proline. Their purification is easy, and a determination of the water of crystallisation and of the copper establishes the identity of the compound. The phenylhydantoine derivative may also be used for this purpose.

Phenylalanine.—Phenylalanine is separated in the form of its ester from those of serine and glutamic and aspartic acids. The mixed esters are dissolved in water, and if a large amount of phenylalanine ester be present, it separates in the form of oily drops, but in any case the aqueous solution is extracted with ether. The ester, obtained after removal of the ether, is hydrolysed by evaporation with concentrated hydrochloric acid, and the resulting phenylalanine hydrochloride is purified by crystallisation from strong hydrochloric acid. By evaporating its aqueous solution with ammonia, treating with ice-cold water to dissolve the ammonium chloride, and precipitating it from its hot aqueous solution by alcohol, a pure preparation of phenylalanine is generally obtained, from the weight of which its percentage amount in the protein is calculated.

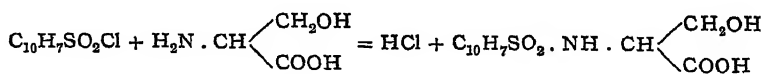
Glutamic Acid.—The greater part of the glutamic acid is isolated as hydrochloride before the mixture of amino acids is esterified. It is contained with aspartic acid ester in the aqueous solution after the phenylalanine ester has been extracted by ether, and it is separated from aspartic acid, after hydrolysis by baryta, by conversion into its hydrochloride, from this it is obtained by treatment with the calculated quantity of soda to combine with the hydrochloric acid and by crystallisation from water, in which it is soluble with some difficulty.

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Elementary analysis of the free acid, or of its hydrochloride, determines its identity and its weight gives the amount in the protein.

Aspartic Acid.—A portion of the aspartic acid, after separation from phenylalanine ester and after hydrolysis by baryta, may separate as barium salt; this is the barium salt of racemic aspartic acid. The remainder is isolated, when the glutamic acid has been removed as hydrochloride, by boiling with lead hydroxide and treating with hydrogen sulphide to remove hydrochloric acid and lead respectively, and by crystallising from water. It may be characterised by conversion into its copper salt, or by analysis, and is estimated by its weight.

Serine.—Its ester is contained in the fractions which distil between 100° and 130° at 0.5 mm. The mixed esters contained in this fraction are treated with a small quantity of water and then with five to six volumes of petroleum ether, which precipitates serine ester as an oil; the oil is then shaken up with petroleum ether to remove admixtures as far as possible and is hydrolysed with baryta water. On removal of the baryta it crystallises when the solution is concentrated, and it is purified by treatment with alcohol, which dissolves other substances which are also present, and recrystallisation from water. Its β -naphthalene sulpho-derivative,



obtained by shaking it in alkaline solution with β -naphthalene sulphochloride, is very suitable for its characterisation.

The Isolation of Oxyproline.

Only in a few cases has this compound been isolated from the products of hydrolysis of proteins, since its separation is extremely laborious. It can only be effected after all the other amino acids have been removed by crystallisation and by the ester method, and after the diamino acids have been precipitated by phosphotungstic acid. From the last mother-liquors it is obtained by crystallisation, and is best identified in the form of its β -naphthalene sulpho-derivative.

The Isolation and Estimation of Tryptophane.

Tryptophane is not obtained in any large amount by the hydrolysis of proteins by acids and is best prepared by the action of trypsin. According to Hopkins and Cole, the protein is digested in alkaline solution by trypsin, until the solution gives a maximal coloration when tested with bromine water; the solution is then acidified, boiled and

filtered. The clear solution (better after concentrating and filtering off tyrosine, which crystallises out) is acidified with sulphuric acid until it contains 5 per cent., and then mercuric sulphate dissolved in 5 per cent. sulphuric acid is added as long as a precipitate, which contains tryptophane, cystine and tyrosine, is formed. The precipitate is freed from tyrosine by washing with 5 per cent. sulphuric acid in which the tyrosine compound is soluble, that is, until the washings no longer react with Millon's reagent. It is then decomposed by sulphuretted hydrogen, and the solution containing cystine and tryptophane is again acidified with sulphuric acid to 5 per cent and fractionally precipitated with the mercuric sulphate reagent. The cystine is thrown down first, and filtered off, and then the tryptophane is precipitated. The precipitate is again decomposed by hydrogen sulphide, and the solution, freed from sulphuric acid, is evaporated down, alcohol being continually added to hasten the evaporation and prevent decomposition of the tryptophane, which is estimated by weighing.

Neuberg and Popowsky, as also Abderhalden and Kempe, have introduced a few alterations in the procedure, such as evaporation *in vacuo*, and Levene and Rouiller suggested in 1906 that the tryptophane, on account of its proneness to decompose on evaporation of its solution with consequent loss, be estimated colorimetrically; the mercury sulphate precipitate is decomposed, and the solution, freed from hydrogen sulphide, is titrated with bromine water in presence of amyl alcohol. Both cystine and tyrosine react with bromine water; the latter can, however, be removed, but for the former a correction has to be made. Up to the present no values concerning the amount of tryptophane in various proteins have appeared, and it will be of interest to see if the values so obtained are very much higher than those obtained by crystallisation of the tryptophane.

II. THE DIAMINO ACIDS.

The separation and estimation of the three compounds—arginine, histidine, lysine—is carried out by the method described by Kossel and Kutscher in 1900, which was slightly modified in 1903 by Kossel and Patten. It is based upon the earlier work of Drechsel, Hedin and Kossel, and depends upon the precipitation of arginine and histidine as their silver salts, and of lysine by phosphotungstic acid, and then by picric acid.

As described by Kossel, Kutscher and Patten the method is as follows:—

- I. About 25 to 50 grammes of protein are hydrolysed by boiling with

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a mixture of three times the weight of concentrated sulphuric acid and six times the weight of water under a reflux condenser for fourteen hours. The exact amount of protein is then estimated by making the volume up to 1 litre, and determining the nitrogen in 5 or 10 c.c. by Kjeldahl's method; from this figure the amount of protein can be calculated, if the amount of nitrogen in it be known.

II. The acid solution is treated with a hot concentrated solution of baryta until the reaction is only faintly acid and almost the whole of the sulphuric acid is precipitated as barium sulphate, which is filtered off and thoroughly washed out with boiling water. The filtrate and washings are evaporated down and again made up to 1 litre. A determination of the nitrogen in 5 or 10 c.c. of this solution gives the amount of nitrogen contained in the melanin, which is carried down by the barium sulphate. It is known as "humins nitrogen I."

In this liquid also two determinations are made of the amount of nitrogen present as ammonia by distilling portions of 100 c.c. with magnesium oxide. From the remainder the ammonia is removed by evaporating with magnesia on the water bath. The three portions, free from ammonia, are then combined, and made alkaline with baryta. The precipitate of barium sulphate is filtered off and thoroughly washed out, the excess of barium removed by dilute sulphuric acid and the precipitate again filtered off and washed out. Filtrate and washings are combined together, evaporated down and made up to 1 litre and a Kjeldahl nitrogen determination again made. Allowing for the nitrogen given off as ammonia, the difference between this and the previous estimation gives the humins nitrogen II. contained in the alkaline barium magnesia precipitate.

III. The solution, now containing a small quantity of sulphuric acid, is placed in a 5-litre flask, made up to 3 litres and heated on the water bath. Finely powdered silver sulphate is slowly added until the solution contains sufficient to give a yellow, not a white, precipitate, when a drop is removed and tested with baryta water in a watch-glass. If, during the process, there be any undissolved silver sulphate at the bottom of the flask it is dissolved by adding more water before a fresh quantity is added, in order that a yellow precipitate be given in the test drop with baryta. As soon as sufficient silver is present to combine with all the arginine and histidine, it is allowed to cool to 40° C. and then saturated with finely powdered baryta. The precipitate which is thus formed, and which consists of the silver salts of arginine and histidine, is filtered off, stirred up with baryta, again filtered off and washed with baryta water.

IV. Separation of arginine and histidine. The above precipitate of the silver salts of these compounds is suspended in water containing sulphuric acid, and decomposed with hydrogen sulphide. The filtrate from the silver sulphide, which is thoroughly extracted with boiling water, is evaporated down to remove the hydrogen sulphide and again made up to 1 litre; a Kjeldahl nitrogen determination in 20 c.c. now gives the amount of nitrogen in the substances precipitated by silver and baryta.

The liquid is now neutralised with baryta, and barium nitrate is added, so long as a precipitate of barium sulphate is formed; this is filtered off and washed. The filtrate is concentrated to 300 c.c. and treated with silver nitrate, as before, till a test drop gives a yellow colour with baryta; when this occurs it is exactly neutralised with baryta, and from a burette small quantities of baryta are added till the silver salt of histidine is completely precipitated; this is determined by taking out a drop when the precipitate has settled and testing with ammoniacal silver solution; if a precipitate easily soluble in excess of ammonia be formed, when the two liquids come together, histidine is still present; and more baryta water must be added, until it is completely thrown out, when it is filtered off, stirred up with water, again filtered off and washed out.

The precipitate of the silver salt of histidine is then stirred up in a measured quantity of 5 per cent. sulphuric acid and decomposed with hydrogen sulphide. The filtrate and washings from the silver sulphide are concentrated so that the solution contains $2\frac{1}{2}$ per cent. sulphuric acid and then precipitated by not too large an excess of mercuric sulphate. The precipitate is allowed to stand for twelve to twenty-four hours, when it is filtered off and decomposed by sulphuretted hydrogen. A nitrogen determination in this solution by Kjeldahl's method gives the amount of nitrogen from which the amount of histidine can be calculated; the histidine itself is obtained by making alkaline with baryta, filtering off the barium sulphate, removing excess of baryta by carbon dioxide, evaporating to dryness, extracting the residue with boiling water, filtering from barium carbonate, adding hydrochloric acid and evaporating down, when histidine hydrochloride $C_6H_9N_3O_2 \cdot 2HCl$ is obtained.

V. The filtrate containing the arginine is saturated with baryta, and the precipitate of the silver salt of arginine, so obtained, is stirred up with baryta, filtered off and washed till free from nitric acid. It is then suspended in water containing sulphuric acid and decomposed with hydrogen sulphide. The filtrate and washings from the precipitate

of silver sulphide are evaporated down and made up to 1 litre; the amount of arginine is calculated from the amount of nitrogen determined in 10 or 20 c.c. of this liquid by Kjeldahl's method. The remainder is freed from sulphuric acid by baryta, the excess of which is removed by carbon dioxide, and the arginine is determined as nitrate $C_6H_{14}N_4O_2 \cdot HNO_3 + \frac{1}{2} H_2O$ by neutralising with nitric acid and evaporating and drying, when it is obtained as a dry white crystalline mass.

VI. The lysine is obtained from the filtrate from the precipitate of arginine and histidine. This is acidified with sulphuric acid, freed from silver by hydrogen sulphide and evaporated to 500 c.c. Sulphuric acid is then added until the content is 5 per cent. and the lysine is precipitated by phosphotungstic acid. The precipitate is filtered off and thoroughly washed, and is decomposed by baryta; the barium phosphotungstate formed is filtered off, and the filtrate, freed from baryta by carbon dioxide, is evaporated almost to dryness; the residue is dissolved in water, filtered from barium carbonate and again evaporated, it is then treated with small quantities of alcoholic picric acid, so long as a precipitate is formed; excess must be avoided as lysine picrate is soluble in excess. After some hours it is filtered off and washed with very little absolute alcohol; it is then dissolved in boiling water and evaporated, when lysine picrate $C_6H_{14}N_2O_2 \cdot C_6H_2(NO_3)_2 \cdot OH$ crystallises out, and is collected on a weighed filter. The mother-liquor yields a little more lysine picrate, which is treated in the same way.

The separation and estimation of the two main groups of amino acids can be carried out in one experiment, instead of separately as described. The protein is hydrolysed by sulphuric acid, the tyrosine, cystine and diaminotrioxydodecanic acid are removed by crystallisation, and the diamino acids are precipitated by phosphotungstic acid. From this precipitate they are obtained by decomposition with baryta, and they are then separated by means of their silver compounds by Kossel, Kustcher and Patten's method. The filtrate from the phosphotungstic acid precipitate is freed from the excess of phosphotungstic acid by means of baryta, and the solution is treated by Fischer's ester method for the monoamino acids.

The combination of the two processes is generally only carried out when the amount of protein available is limited; they require very different quantities of material; thus, the diamino acids can be determined in 25 to 50 grammes of protein with considerable accuracy, whereas the monoamino acids can only be determined with fair accuracy when 250 to 500 grammes of protein can be used. On the whole, it is not

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advisable to combine the processes, since the phosphotungstic acid precipitation does not effect a perfect separation of the two groups.

A very large number of proteins have now been hydrolysed and the products of hydrolysis determined by these methods by investigators not only in Germany, but also in France and America. The results are collected together in the following tables which are arranged according to the classification of the proteins.

PROTAMINES.

	Salmine. (Kossel, Abderhalden; Kossel and Dakin)	Sturine (Kossel and Kutscher)	Clupeine. (Kossel and Kutscher, Kossel and Dakin)	Scombrine (Kossel)	Cyclo- terine. (Kossel and Kutscher, Kossel, Morkowin)	Cyprinine I. (Kossel and Dakin.)	Cyprinine II
Glycine	
Alanine . .			+	.		.	
Valine . .	4.3		+	.		.	+
Leucine	
Isoleucine	
Phenylalanine .				.			
Tyrosine . .					8.3	+ ?	+
Serine . .	7.8		+	.			.
Cystine
Proline . .	11.0		+	.	.		.
Oxyproline		
Aspartic Acid .	.				.		
Glutamic Acid .					.		
Tryptophane	+	+		.
Arginine . .	87.4	58.2	82.2	+	62.5	4.9	+
Lysine . .	0	12.0	0	0	0	28.8	+
Histidine . .	0	12.9	0	0	0	0	0
Diaminotrioxo- dodecanic Acid	.				..		
Ammonia		
Total	110.5	83.1	82.2	.	70.8	33.7	

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HISTONES

	Globin of Hæmoglobin from Horse's Blood. (Abderhalden)	Globin of Hæmoglobin from Dog's Blood (Abderhalden and Baumann)	Thymus-Histone (Kossel and Kutscher, Kutscher, Abderhalden and Rona)		Lota-Histone (Ehrstrom)	Gadus-Histone (Kossel and Kutscher)
Glycine	0.5	.	.
Alanine .	4.19	3.0	.	3.5	..	.
Valine .	.	1.0
Leucine .	29.04	17.5	.	11.8	.	.
Isoleucine
Phenylalanine.	4.24	5.0	.	2.2	.	.
Tyrosine .	1.33	.	6.4	5.2	.	.
Serine .	0.56
Cystine .	0.31
Proline .	2.34	4.5	.	1.5	.	.
Oxyproline .	1.04
Aspartic Acid .	4.43	2.5	.	0	.	.
Glutamic Acid	1.73	1.2	3.7	0.5	..	.
Tryptophane .	+
Arginine .	5.42	.	14.4	15.5	12.0	15.6
Lysine .	4.28	.	7.7	6.9	3.2	8.3
Histidine .	10.96	.	1.3	1.5	2.9	2.4
Diaminotrioxo- dodecanic Acid
Ammonia .	.	.	1.7	.	0.7	0.8
Total .	69.87	34.7	35.2	49.1	18.8	27.1

ALBUMINS.

	Egg-albumin (Abderhalden and Pregl, Mörner)	Serum-albumin (Abderhalden, Mörner)	Lact-albumin. (Abderhalden and Pribram)	Bence-Jones Albumin ¹ (Abderhalden and Rostowski)
Glycine .	0	0	0	1.7
Alanine .	2.1	2.7	2.5	4.5
Valine	0.9	.
Leucine .	6.1	20.0	19.4	10.6
Isoleucine
Phenylalanine	4.4	3.1	2.4	1.5
Tyrosine .	1.1	2.1	0.9	1.7
Serine .	.	0.6	.	..
Cystine .	0.3	2.5	..	.
Proline .	2.3	1.0	4.0	1.9
Oxyproline
Aspartic Acid	1.5	3.1	1.0	4.5
Glutamic Acid	8.0	7.7	10.1	6.0
Tryptophane	+	+	.	..
Arginine
Lysine
Histidine
Diaminotrioxo- dodecanic Acid
Total .	25.8	42.8	41.2	32.4

¹ This protein is included under this group on account of its name; no classification has yet been given to it, though from its reactions it is more closely allied to the proteoses.

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GLOBULINS.

	Serum-globulin (Abderhalden, Abderhalden and Samuely, Morner)		Fibrin (Abderhalden and Vaitinovici, Morner.)
Glycine . . .	3 5	.	3'0
Alanine . . .	2 2	.	3'6
Valine . . .	+	.	1'0
Leucine . . .	18 7	.	15 0
Isoleucine
Phenylalanine . . .	3 8	.	2 5
Tyrosine . . .	2 5	.	3 5
Serine	0 8
Cystine	0'7	1'5	1 1
Proline	2 8	..	3 6
Oxyproline
Aspartic Acid	2'5	.	2'0
Glutamic Acid	8'5	.	10 4
Tryptophane	+	.	+
Arginine
Lysine
Histidine
Diaminotrioxydodecanic Acid
Total	45'2		46 5

CRYSTALLISED VEGETABLE GLOBULINS.

	Edestin from Hemp Seed (Abderhalden, Kossel and Patten, Schulze and Winterstein)			Edestin from Cotton Seed (Abderhalden and Rostoski)	Edestin from Sunflower Seed (Abderhalden and Reinhold)	Cryst Globulin from Pumpkin Seed (Abderhalden and Berghausen)	Cryst Globulin from Squash Seed (Osborne and Clapp, Osborne and Gilbert)	Excelsin from Brazil Nut (Osborne and Clapp)
Glycine . . .		3 8	..	1'2	2 5	0 1	0 6	0 6
Alanine . . .		3 6		4'5	4 5	+	1 9	2'3
Valine . . .		+		+	0 6	0'7	0'3	1'5
Leucine . . .		20'9		15 5	12'9	4'7	7 3	8 7
Isoleucine	
Phenylalanine . . .		2'4	...	3 9	4 0	2'6	3'3	3 5
Tyrosine	2'1	.	2 3	2'0	1'4	3 1	3'1
Serine . . .		0 4		0'4	0 2			
Cystine	0 3			.		0 3	
Proline	1'7	.	2 3	2 8	1'7	2'9	3 6
Oxyproline . . .		2 0		.	.		.	
Aspartic Acid . . .		4'5	.	2 9	3 2	4'5	3 3	3'8
Glutamic Acid	6'3		17 2	13'0	13'4	12'4	12 9
Tryptophane . . .		+	..	+	+	..	+	+
Arginine . . .	11 2	11'7	14 4	14 4	16'1
Lysine . . .	1'6	1'0	1'7	2'0	1'6
Histidine . . .	1 4	1'1	2'4	2'6	1'5
Diaminotriox- y-dodecanic Acid
Ammonia	1 6	1'8
Total . . .		61 8	...	50 2	45 7	29 1	56'0	61'0

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GLUTELINS.

	Legumin, from Pea (Osborne and Clapp)	Amandin, from Almond (Osborne and Clapp , Osborne and Gilbert)	Glycinin, from Soy Bean (Osborne and Clapp)	Phaseolin, from White Kidney Bean (Osborne and Clapp)	Protein of Maize (Osborne and Clapp)
Glycine	0 38	0 51	0 97	0 6	0 3
Alanine	2 03	1 40		1 8	
Valine	0 16	0 68	1 1	
Leucine	8 00	4 45	8 45	9 7	6 2
Isoleucine			
Phenylalanine	3 75	2 53	3 86	3 3	1 8
Tyrosine	1 55	1 12	1 86	2 2	3 8
Serine	0 53	?	.	0 4	.
Cystine
Proline	3 22	2 44	3 78	2 8	5 0
Oxyproline
Aspartic Acid	5 30	5 42	3 89	5 3	0 7
Glutamic Acid	13 80	23 14	19 46	14 6	12 7
Tryptophane	+	+			+
Arginine	10 12	11 85	5 12	4 9	7 1
Lysine	4 29	0 70	2 71	4 0	3 0
Histidine	2 42	1 58	1 39	2 0	3 0
Diaminotrioxydodecanic Acid
Ammonia	1 99	3 70	2 56	2 1	2 1
Total	57 43	59 00	54 73	54 8	45 7

GLUTELINS

	Protein, from Fir-tree Seed (Abderhalden and Teruuchi, Schulze and Winterstein)	Conglutin, from Lupine Seed (Abderhalden and Herrick, Schulze and Winterstein)	Legumin, from Bean (Abderhalden and Bakkin, Schulze and Winterstein.)	Avenin, from Oats (Abderhalden and Hamalainen)	Gluten, from Wheat (Abderhalden and Malengreau, Kossel and Kutscher)
Glycine	0 6	0 8	1 0	1 0	0 4
Alanine	1 8	2 5	2 8	2 5	0 3
Valine	+	1 1	1 0	1 8	..
Leucine	6 2	6 8	8 2	15 0	4 1
Isoleucine	
Phenylalanine	1 2	3 1	2 0	3 2	1 0
Tyrosine	1 7	2 1	2 8	1 5	1 9
Serine	0 1	+	
Cystine	0 3	
Proline	2 8	2 6	2 3	5 4	4 0
Oxyproline
Aspartic Acid	1 8	3 0	4 0	4 0	0 7
Glutamic Acid	7 8	19 5	16 3	18 4	24 0
Tryptophane	+	+	+
Arginine	10 9	6 9	4 6	...	4 4
Lysine	0 3	2 1	5 1	..	2 2
Histidine	0 7	0 7	1 1	..	1 2
Diaminotrioxydodecanic Acid
Ammonia	2 5
Total	36 2	51 2	51 2	52 8	46 7

GLIADINS.

	Gliadin Wheat (Abderhalden and Samuely, Kossel and Kutscher)	Gliadin, from Wheat (Osborne and Clapp)	Gliadin, from Rye (Osborne and Clapp)	Hordein, from Barley (Osborne and Clapp)	Hordein (Klein- schmitt)	Zein, from Maize (Osborne and Clapp)	Zein Maize (Langstein, Kutscher, Kossel and Kutscher)
Glycine .	0.7	0.02	0.13	0.00	0	0.00	—
Alanine .	2.7	2.00	1.33	0.43	1.4	2.23	0.5
Valine .	0.4	0.21	0.13	0.13	1.4	0.29	+
Leucine .	6.0	5.61	6.30	5.67	7.0	18.60	11.2
Isoleucine
Phenylalanine .	2.6	2.35	2.70	5.03	5.5	4.87	7.0
Tyrosine .	2.4	1.20	1.19	1.67	4.0	3.55	10.1
Serine .	0.2	0.13	0.06	.	0.1	0.57	—
Cystine .	.	0.45
Proline .	2.4	7.06	9.82	13.73	5.9	6.53	1.5
Oxyproline
Aspartic Acid .	1.3	0.58	0.25	.	1.3	1.41	1.0
Glutamic Acid	31.5	37.33	33.81	36.35	41.3	18.28	11.8
Tryptophane	1.0	+	+	+	.	0.00	.
Arginine .	2.8	3.16	2.22	2.16	3.2	1.16	1.9
Lysine .	0.0	0.00	0.00	0.00	0.0	0.00	0.0
Histidine .	1.2	0.61	0.39	1.28	0.5	0.43	0.9
Diaminotriox- y-dodecanic Acid
Ammonia .	4.1	5.11	5.11	4.87	4.4	3.61	2.6
Total	59.3	65.81	64.31	71.32	76.0	61.53	48.5

PHOSPHOPROTEINS

	Caseinogen, Cow's Milk (Abderhalden, Fischer, Mörner, Fischer and Abderhalden, Hart)	Caseinogen, Goat's Milk (Abderhalden and Schittenhelm)	Caseinogen, Human Milk (Abderhalden and Schittenhelm)	Vitelin (Abderhalden and Hunter)	Vitelin (Levene and Alsberg)	Vitelin (Hugounenq)
Glycine .	0	0	.	1.1	trace	<0.5
Alanine .	0.9	1.5	.	+	0.2	<0.5
Valine .	1.0	2.4	..	1.5
Leucine .	10.5	7.4	.	11.0	3.3	6.8
Isoleucine
Phenylalanine	3.2	2.75	.	2.8	1.0	0.7
Tyrosine .	4.5	4.95	4.71	1.6	0.4	2.0
Serine .	0.23	..	.	—	.	<0.5
Cystine .	0.06
Proline .	3.1	4.6	.	3.3	4.0	<0.5
Oxyproline .	0.25
Aspartic Acid .	1.2	1.2	.	0.5	0.6	0.7
Glutamic Acid	11.0	12.0	.	12.2	1.0	1.0
Tryptophane .	1.5	+
Arginine .	4.84	1.2	1.0
Lysine .	5.80	2.4	1.2
Histidine .	2.59	trace	2.1
Diaminotriox- y-dodecanic Acid	0.75	+
Ammonia .	1.6	1.2
Total	53.42	34.4	...	34.9	14.1	20.2

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SCLEROPROTEINS.

	Gelatin (Fischer, Levene and Aders, Fischer, Hart, Kossel and Kutscher)		Silk- gelatin (Fischer and Skita, Fischer)	Silk- fibroin (Fischer and Skita, Fischer)	Spider- silk- fibroin (Fischer)	Elastin (Abderhalden and Schitten- helm, Schwarz, Kossel and Kutscher)	Spongin (Abderhalden and Strauss, Kossel and Kutscher)	Koilin (Knauff- Lenz)	Egg- membrane of Scyllium Stellare (Pregl)
Glycine . . .		16.5	0.2	36.0	35.2	25.8	13.9		2.6
Alanine . . .		0.8	5.0	21.0	23.4	6.6			3.2
Valine . . .		1.0		0		1.0			
Leucine . . .		2.1		1.5	1.8	21.4	7.5		5.8
Isoleucine . .		—							
Phenylalanine .		0.4		1.5		3.9			3.3
Tyrosine . . .		0	5.0	10.5	8.2	0.4	0		10.6
Serine . . .		0.4	6.6	1.6					
Cystine . . .		—							?
Proline . . .		5.2		+	3.7	1.7	6.3		4.4
Oxyproline . .		3.0							
Aspartic Acid .		0.6		+		+	4.7		2.3
Glutamic Acid .		0.9		0	11.7	0.8	18.1		7.2
Tryptophane . .		0							+
Arginine . . .	9.3	7.6	+	1.0	5.24	0.3		3.6	3.2
Lysine . . .	5	2.8		+				1.7	3.7
Histidine . . .		0.4	4.0	+				0.1	1.7
Diaminotrioxo- dodecanic Acid .		—							..
Ammonia	0.4			1.2				...
Total . . .		42.1	20.8	73.1	90.44	61.9	50.5	5.4	48.0

SCLEROPROTEINS

	Keratin, from Ox Horn (Fischer and Dörping- haus, Mörner)	Keratin, from Sheep's Horn (Abderhal- den and Voitinovici)	Keratin, from Sheep's Wool (Abderhal- den and Voitinovici)	Keratin, from Horse Hair. (Abderhal- den and Wells)	Keratin, from Goose Feathers (Abderhal- den and Le Count)	Keratin, from Egg- membrane (Abderhal- den and Ebstein, Mörner)	Keratin, from Egg- membrane of Testudo Graeca. (Abderhal- den and Strauss)	Ichthyle- pidin, from fish Scales (Abderhal- den and Voitinovici)
Glycine . . .	0.4	0.5	0.6	4.7	2.6	3.9	+	5.7
Alanine . . .	1.2	1.6	4.4	1.5	1.8	3.5	+	3.1
Valine . . .	5.7	4.5	2.8	0.9	0.5	1.1	...	
Leucine . . .	18.3	15.3	11.5	7.1	8.0	7.4		15.1
Isoleucine							
Phenylalanine .	3.0	1.9		0	0		+	
Tyrosine . . .	4.6	3.6	2.9	3.2	3.6			1.0
Serine . . .	0.7	1.1	0.1	0.6	0.4			
Cystine . . .	6.8	7.5	7.3	{ above }		7.6		
Proline . . .	3.6	3.7	4.4	3.4	3.5	4.0	11.8?	6.7
Oxyproline						
Aspartic Acid .	2.5	2.5	2.3	0.3	1.1	1.1	1.8?	1.2
Glutamic Acid .	3.0	17.2	12.9	3.7	2.3	8.1	3.0?	9.2
Tryptophane	
Arginine . . .	2.3	2.7	
Lysine	0.2	
Histidine				
Diaminotrioxo- dodecanic Acid
Total . . .	52.1	62.3	49.2	35.4	23.8	36.7	16.6?	42.0

DERIVATIVES OF PROTEINS.

	Syntonin. (Abderhalden and Sasaki, Hart)	Hetero- albumose, from Syntonin (Hart.)	Prot- albumose, from Syntonin (Hart)	Deutero- albumose, from Witte's Peptone (Haslam)	Hetero- albumose, from Witte's Peptone (Haslam)	Protein in Urine (Abder- halden and Pregl)
Glycine	05			.	.	+
Alanine	40			..		+
Valine	09					
Leucine	78			.		+
Isoleucine				
Phenylalanine .	25					
Tyrosine	22	
Serine				
Cystine				
Proline	33	
Oxyproline	
Aspartic Acid .	05					+
Glutamic Acid .	136					+
Tryptophane . .				.		
Arginine	51	85	46	71	49	.
Lysine	33	71	31	69	35	
Histidine . . .	27	04	34	15	22	
Diaminotrioxido- caneic Acid . .						
Ammonia	09	10	08	10	08	..
Total	473	170	119	165	114	

The result of hydrolysis definitely shows that the various proteins are composed of the same units, in some cases certain are missing, and in other cases one or more units exceed the others by very large amounts.

The protamines are built up almost exclusively of diamino acids, salmine containing over 80 per cent. of arginine. Only small amounts of monoamino acids are present in them, and even these amounts may be due to impurity, for fish sperm only at maturity is made up of protamine and nucleic acid, whereas at other times histone takes the place of protamine, and histones contain less diamino acids. Kossel and Dakin's analysis appears to show us a quantitative result in the case of salmine.

The histones contain about 30 per cent. of diamino acids, and only in the case of thymus-histone has an estimation been made of the monoamino acids. They were supposed to be intermediate compounds between protamines and other proteins, and this supposition is confirmed by the results of analysis.

The protein constituent—globin—of hæmoglobin has always been regarded as a histone, but the presence of only 20 per cent. of diamino

acids is against this supposition. Further, the principal diamino acid is histidine, whereas in the other histones it is arginine. It should be noted that hæmoglobin contains a considerably greater amount of histidine than the other proteins.

Albumins contain no glycine, whereas globulins contain this amino acid. Their differentiation on physical grounds is thus borne out by chemical analysis.

No great difference is to be noted between the crystalline vegetable globulins and the vegetable proteins soluble in dilute alkali, but there is a marked difference between these proteins and the alcohol-soluble vegetable proteins. These contain scarcely any glycine, the small amount obtained is probably due to impurity; except in zein the amount of leucine in them in comparison with other proteins is small. The most distinct features of these proteins are the absence of lysine and the presence of an enormous quantity of glutamic acid (except zein); the amount of proline is also high, and the amount of arginine is low. It is curious that those amino acids which are absent in zein are present in the protein of maize, which is soluble in dilute alkali. The mixture of these proteins in the grain, therefore, gives all the amino acids present in other proteins. As the gliadins are all so much alike, it would seem that they are the same protein.

There is no striking peculiarity noticeable with the phosphoproteins; the two caseinogens examined appear to have the same composition. Vitellin, which has been hydrolysed by three sets of investigators, has given very different results; the most detailed examination is that by Hugounenq, whose values are lower than those of Abderhalden and Hunter and of Levene and Alsberg; the latter observers used a purified product, whereas the former used the commercial article. The amount of glycine is small in vitellin and it corresponds in this particular with caseinogen.

The scleroproteins, which represent a heterogeneous collection of proteins in their physical properties give on hydrolysis, as would be expected, results which support their classification.

Gelatin contains no tryptophane, cystine or tyrosine, but it contains a large amount of glycine and a considerable amount of proline. It appears to have no similarity to silk-gelatin, which contains so much serine.

Silk-fibroin is composed of practically only three amino acids, glycine, alanine and tyrosine, and is probably the simplest protein known. It resembles the fibroin of spider's silk very closely, but here the presence of so much glutamic acid is one of the characteristics.

Elastin again seems to be made up of only three or four amino acids; spongin is rather more complex.

The keratins are distinguished by containing more cystine than any other protein; in human hair it exists to the extent of about 14 per cent. (Mörner). Tyrosine also is present in fair quantities

The presence of diamino acids in all proteins led Kossel to suppose that there was a protamine nucleus (*i.e.*, of diamino acids) in all proteins; the more recent work, especially that by Osborne and Clapp on the gliadins, where the diamino acids are present in such small amounts, though it supports the theory, yet suggests that proteins may exist in which it is not present, more especially if the view of Emil Fischer be taken that all the proteins we know, even the crystalline ones, are still mixtures of several proteins. The isolation of complexes containing only diamino acids from proteins, where they are combined together, will be the only proof of a protamine nucleus in a protein molecule.

SECTION II

THE CHEMICAL CONSTITUTION OF ITS UNITS, OR THE DISCOVERY AND SYNTHESSES OF THE AMINO ACIDS.

IN Section I. an account was given of how the units of the protein molecule are now isolated and estimated, and the results were embodied in several tables. In general, it may be said, that the amino acids were first discovered in that protein in which they occurred in largest amounts. An account will be given in this section of the discovery and of the determination of the constitution of each amino acid.

A. MONOAMINO-MONOCARBOXYLIC ACIDS.

Glycine

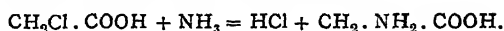
This, the simplest of the products of hydrolysis of the proteins, was also the first to be discovered; it was obtained by Braconnot, in 1820, by boiling gelatin with dilute sulphuric acid, and on account of its sweet taste he called it sugar of gelatin. In 1846 Dessaignes obtained it from hippuric acid by hydrolysis, and, in 1848, Strecker showed that cholic acid (now glycocholic acid) consisted of this amino acid and cholalic acid, so that, as a constituent of substances of animal origin, it became of great importance. Its presence in elastin was demonstrated by Jeanneret, in horn by Horbaczewski, in spongin by Krukenberg, in conchiolin by Wetzel, and in silk-fibroin by Cramer; Faust and Spiro showed that it was present in globulin. It does not occur in albumin, nor in caseinogen, nor in hæmoglobin; it is present only to a small extent in the vegetable proteins, and for this reason it was not isolated until Abderhalden showed its presence in these proteins by Fischer's ester method.

In the free state, glycine was found by Chittenden in an extract of the American mussel, *Pecten irradians*, and of recent years it has been recorded as sometimes occurring in the urine.

Its elementary composition of $C_2H_5NO_2$ was first correctly determined in 1846 by Mulder and by Laurent, and in this year, after Dessaignes had pointed out the unsuitability of the name given to it of

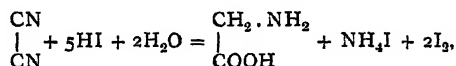
sugar of gelatin, as there were other substances like it with a sweet taste and which were not fermentable, its name of glycoll (γλυκύς, sweet, κολλα, glue) originated and was first used by Horsford, who made an extensive study of it and its derivatives, whilst working in Liebig's laboratory where much of the early work on proteins was carried out.

Laurent regarded glycoll as belonging to the ammonia type of organic compounds; it was supposed by Cahours to be a derivative of acetic acid, which supposition was only proved by its synthesis from bromoacetic acid and ammonia by Perkin and Duppa, and from chloroacetic acid and ammonia by Cahours, both in 1858:—

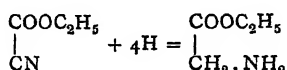


About this time the terms glycine and glycine were used for glycoll as it was then recognised as a homologue of alanine and leucine. The whole of this series of compounds were termed the glycines.

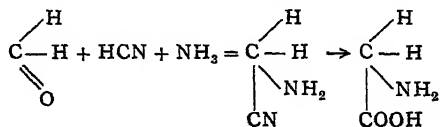
A very interesting synthesis of glycine was described by Emmerling, in 1873, by the action of hydriodic acid upon cyanogen; here the hydriodic acid acts both as a reducing agent and as a hydrolysing agent:—



and, in 1877, Wallach obtained it by the reduction of cyanoformic ester with zinc:—



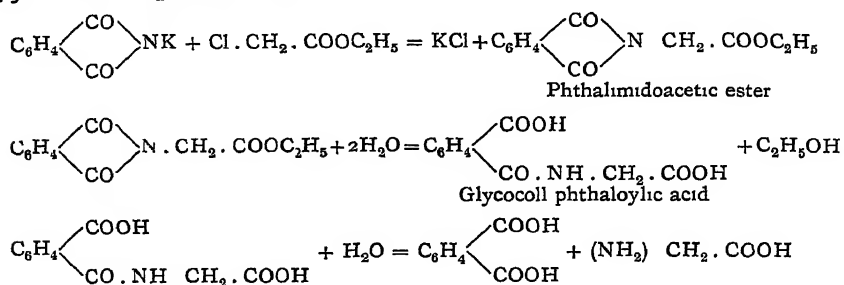
Lubavin, in 1882, stated that glycine was formed by the action of ammonium cyanide upon glyoxal, which probably first breaks down into formaldehyde and then by the cyanhydrin reaction yields glycine:—



The direct synthesis of glycine from formaldehyde was only carried out in 1894 by Eschweiler. This method, as well as the method from chloroacetic acid and ammonia, by which both Nencki and Mauthner and Suida by slight modifications in technique attempted to obtain larger yields, only gives about 20 per cent., but the method described by Gabriel and Kroseberg, in 1889, who made use of Gabriel's phthalimide reaction, as first shown by Goedeckemayer, gives an almost theoretical yield of glycine; this reaction takes place in the following stages:—

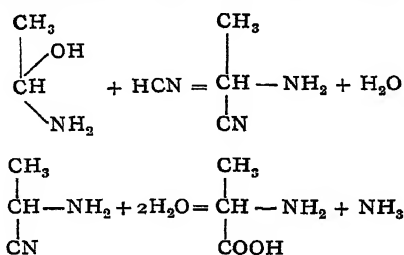
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Phthalimidoacetic ester is obtained by the action of chloracetic ester upon potassium phthalimide, this is first hydrolysed by alkali to glycoll phthaloylic acid, and then by 20 per cent. hydrochloric acid to glycoll and phthalic acid:—



Alanine.

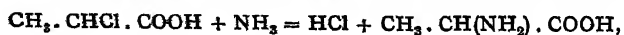
Of the naturally occurring amino acids alanine only was prepared synthetically many years before it was discovered as a constituent of the protein molecule. Its name was given to it by its discoverer, Strecker, who prepared it in 1850 from aldehyde ammonia, which, when treated with hydrogen cyanide gives the aminocyanohydrin, and this by hydrolysis is then converted into the amino acid:—



Owing to the ease with which the aldehyde resinifies in presence of alkali and potassium cyanide the yield of alanine is very poor. If, however, the reaction be carried out in the presence of excess of ammonium chloride and if the potassium cyanide be slowly added to the aldehyde dissolved in ether, a yield of alanine amounting to 60 to 70 per cent. can be obtained, as has been recently shown by Zelinsky and Stadnikoff.

This is the first of the general methods employed in the synthesis of the amino acids.

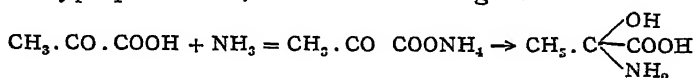
Alanine was prepared in 1860 by Kolbe by the second general method, by the action of ammonia upon α -chloropropionic acid:—



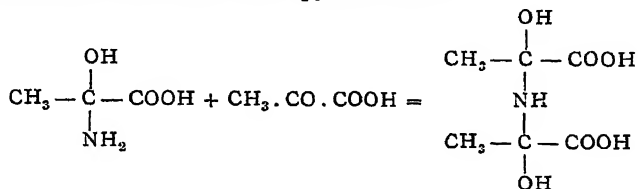
and in 1864 by Kekulé from monobromopropionic acid and alcoholic ammonia.

A synthesis of acetylalanine, from which alanine can be obtained by hydrolysis, was described in 1900 by de Jong. Pyruvic acid was neutralised with ammonium carbonate; there was a considerable rise in temperature, carbon dioxide was evolved and the ammonium salt of acetylalanine crystallised out. The explanation of this reaction is based upon Erlenmeyer and Kunlin's synthesis of phenylalanine from phenylpyruvic acid and it proceeds as follows:—

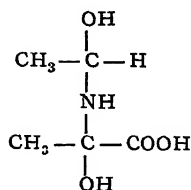
Ammonium pyruvate, which is first formed, is tautomeric with α -amino-oxypropionic acid, into which it changes:—



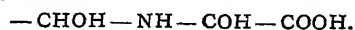
This compound then reacts with pyruvic acid:—



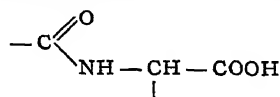
and the compound thus formed loses carbon dioxide giving the compound,



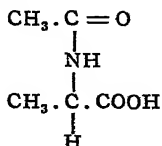
which possesses the group,



By intramolecular rearrangement and loss of water this becomes



The above compound by rearrangement and loss of water is thus converted into acetylalanine:—



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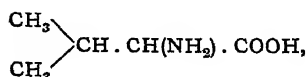
The occurrence of alanine in proteins was first shown by Schutzenberger, who did not actually identify his product with the synthetical one, Weyl in 1881 obtained it as a decomposition product of silk and showed that his preparation was similar in properties to Strecker's synthetical alanine. He thus established it as a constituent of a protein molecule. The researches of Emil Fischer have shown that alanine is a constant constituent of all proteins. It is worthy of note that of the eighteen definitely determined units of a protein molecule, six of them, namely, isoleucine, phenylalanine, tyrosine, serine, histidine and tryptophane, are derivatives of α -aminopropionic acid.

Valine.

A body of the composition $C_5H_{11}NO_2$ was obtained in 1856 by v. Gorup-Besanez from an aqueous extract of pancreas, and on account of its similarity in properties to leucine he regarded it as a homologue of leucine and termed it butalanine. Schutzenberger, in 1879, also obtained a substance which had this empirical formula and properties like that of leucine.

An aminovalerianic acid was described in 1883 by Schulze and Barbieri as occurring in the seedlings of yellow lupines, and subsequently Schulze again isolated it from the extracts of other seedlings. It appeared to correspond to *n*-aminovalerianic acid, which had been synthesised by Lipp.

In 1899 Kossel isolated a similar substance from the protamine, clupeine, of herring milt, and since then E. Fischer and his pupils have obtained it from caseinogen, horn and other proteins. The preparation from horn, when racemised, corresponded in properties with the synthetical α -aminoisovalerianic acid,



which had been first prepared by Clark and Fittig in 1866 from the corresponding bromo-compound and later by Lipp in 1880 from isobutyraldehyde; its derivatives were identical with those of this acid which were prepared by Slimmer in 1902. The exact identity of the natural and synthetical substances was only established in 1906 when Fischer prepared *d*-aminoisovalerianic acid from the synthetical product, and showed that its specific rotation was the same as that of Schulze and Barbieri's natural substance. The name valine was given to this compound in 1906 by E. Fischer.

Leucine.

A substance, corresponding to our leucine, was described by Proust in 1818 under the name of oxide-caséux. Two years later, in 1820, Braconnot isolated from the products resulting by boiling meat with dilute sulphuric acid a substance which he named leucine on account of its glistening white (*λευκος*) appearance. Mulder, in 1839, obtained it by boiling meat with alkali and by the putrefaction of casein. Its occurrence and oxidation products were investigated by Liebig, who regarded it as one of the constituents of the protein molecule, as was proved in 1849 by Bopp, who prepared it from caseinogen, fibrin and albumin by fusion with potash, by hydrolysis with acids and by putrefaction. Hinterberger showed that it was present in horn, and Zollikofer in elastin.

Leucine also occurs in the free state in the various organs of the animal body as pointed out by Frerichs and Stadelé and many other observers.

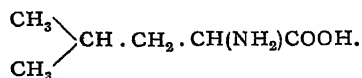
Not only is it present in the animal proteins, but also in the vegetable ones, from which it passes by the action of enzymes into the extracts of germinating seedlings, as shown by Schulze and his co-workers. Leucine is, with the exception of arginine, the most widespread of all the amino acids which go to make up the protein molecule.

Its correct empirical formula $C_6H_{13}NO_2$ was first given to it by Laurent and Gerhardt. These observers and also Cahours showed that it belonged to the glycine series of compounds; Liebig and others showed that on oxidation it gave ammonia and valerianic acid, and also valeronitrile, and Strecker obtained leucic acid by treating it with nitrous acid. But only in 1868, when Hufner obtained caproic acid and ammonia by reducing it with hydriodic acid, was it shown to be an α -aminocaproic acid. Hufner tried to prove this by comparing the natural leucine with two synthetical leucines, (1) that prepared by the action of ammonia on bromocaproic acid obtained from the fermentation caproic acid, and (2) that prepared from isovaleraldehyde, hydrogen cyanide and ammonia which had been first synthesised by Limpricht in 1855. Neither of these two synthetical leucines corresponded exactly with natural leucine, and Hufner, rather than regard them as isomers, regarded them as identical compounds.

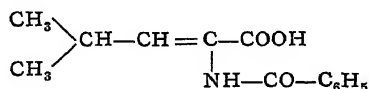
The question of the constitution of leucine was again taken up in 1891 by Schulze and Likiernik. The natural product is optically active, but by heating with baryta at 160° C. it is racemised; this inactive leucine, on being compared with the leucine prepared from

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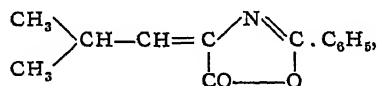
isovaleraldehyde, hydrogen cyanide and ammonia, was found to be identical with it, and further, both compounds gave d-leucine, when acted upon by the mould *Penicillium glaucum*, and the same leucic acid, when treated with nitrous acid. Leucine is therefore α -aminoisobutylic acid,



Among the syntheses of α -amino acids carried out by E. Erlenmeyer jun., by his method, that of leucine was described in 1901 by Erlenmeyer and Kunlin. It was prepared from α -benzoylamido- β -isopropylacrylic acid,

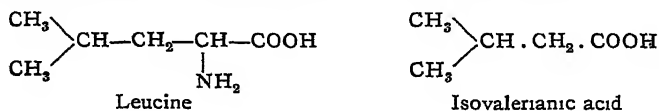


which resulted when isobutylaldehyde and hippuric acid were condensed together in the presence of acetic anhydride and the condensation product,



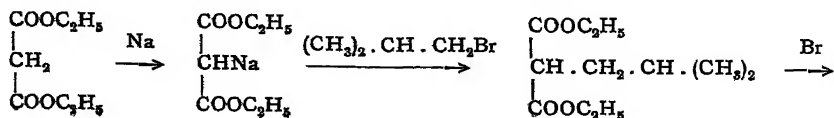
was treated with alkali.

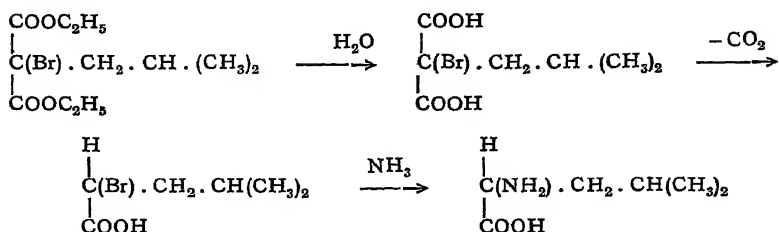
By heating α -benzoylamido- β -isopropylacrylic acid in sealed tubes at 150-170° C., with excess of ammonia, hydrolysis occurred with the formation of leucine, isovalerianic acid and benzoic acid:—



Bouveault and Locquin have also synthesised leucine by the reduction of α -oximinoisobutylic acid, which was prepared in a similar way to the isomeric compound from which they obtained isoleucine.

The most convenient method of preparing leucine by synthesis is that given by Fischer and Schmitz in 1906 by the action of ammonia upon the corresponding halogen derivative of isocaproic acid, which they prepared by brominating the alkylmalonic ester and heating whereby it was converted into the bromo-fatty acid. The several reactions are represented by the following scheme:—

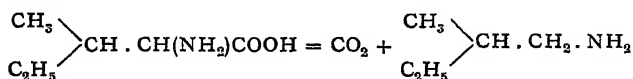


*Isoleucine.*

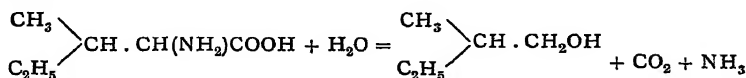
This amino acid was first obtained by F. Ehrlich in 1903 from the nitrogenous constituents of beet-sugar molasses, and was subsequently isolated by him from the decomposition products of fibrin and other proteins. Like leucine, to which it is very similar in properties, it thus appears to be a widely distributed constituent of the protein molecule.

Of the various isomeric amino-caproic acids only leucine and isoleucine occur in the protein molecule; both of them, combined with tyrosine and valine in the form of polypeptides, from which they are easily split off by enzymes, seem to form a very important part of most proteins.

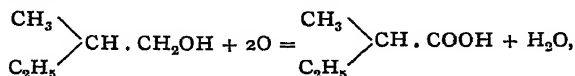
Ehrlich showed that leucine, when heated to 200° C., was converted into d-amylamine with loss of CO₂:—



and that, when fermented by yeast in the presence of cane sugar, it yielded d-amylalcohol:—



This was determined by oxidising to methylethylacetic acid:—

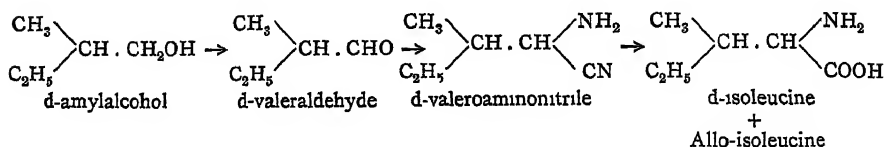


from which the constitution of isoleucine appeared to be α -amino- β -methyl- β -ethyl-propionic acid.

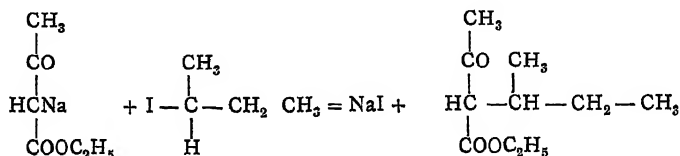
Ehrlich proved this by synthesising it from d-amylalcohol; this was first oxidised to valeraldehyde, which on treatment with hydrogen cyanide and ammonia gave valeroaminonitrile, and then, on hydrolysis d-isoleucine mixed with allo-isoleucine; isoleucine has two asymmetric carbon atoms, and allo-isoleucine is formed by the rearrangement of the groups attached to one of them. By heating natural isoleucine with

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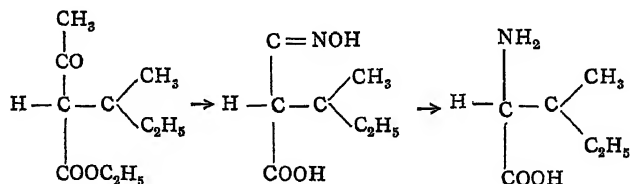
baryta water under pressure, it was found to also undergo a rearrangement, and the product seemed identical with that obtained by synthesis:—



Further proof that isoleucine has this constitution was given by Bouveault and Locquin in 1906. They synthesised it from sec. butyl-acetoacetic ester, which they prepared from sec butyl iodide and sodium-acetoacetic ester:—

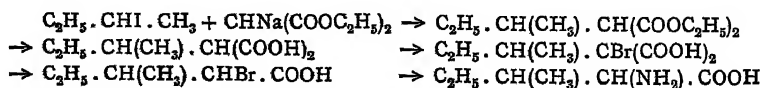


This compound, when treated with nitrosyl sulphate was decomposed into α -oximino-sec-butylic acid, which, on reduction with zinc dust and hydrochloric acid in alcohol, gave a 60 per cent. yield of dl-isoleucine:—



Locquin has since obtained d-isoleucine from this racemic compound which was identical with Ehrlich's natural product, and this therefore has the above constitution.

By the same series of reactions which Fischer and Schmitz employed in the preparation of leucine, F Ehrlich synthesised isoleucine in 1908 from malonic ester and secondary butyl iodide, *i.e.*, according to the following scheme:—



The same synthesis has also been carried out by Brasch and Friedmann.

Phenylalanine.

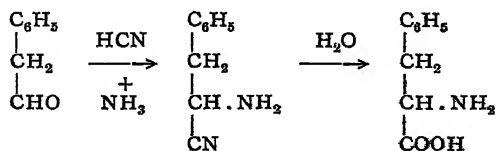
In a note published in 1879 Schulze mentioned a substance which he had obtained from the seedlings of *Lupinus luteus*; two years later he and Barbieri showed that this substance had the composition $C_9H_{11}NO_2$, and they described it as phenylamidopropionic acid, because, on oxidation, it gave benzoic acid, and, when heated, it lost carbon dioxide and gave a base, $C_8H_{11}N$. In its properties it closely resembled Tiemann's phenyl-aminoacetic acid, and they regarded it therefore as a homologue of this acid, though it differed from the substance described by Posen as phenyl-*a*-aminopropionic acid.

About the same time Schutzenberger obtained a substance, which he called tyroleucine, by the action of baryta on proteins; when heated it gave a sublimate of aminovalerianic acid and a base $C_8H_{11}N$, which probably had as mother-substance the same body which was isolated by Schulze and Barbieri.

Schulze, Barbieri and Bosshard next showed that their substance arose during the germination of the seed, and that it was also obtained from vegetable proteins by hydrolysis, by hydrochloric acid and zinc chloride, or by baryta. It was therefore contained in the protein molecule.

It had been known for a long time that benzaldehyde and benzoic acid were formed by the oxidation of animal proteins, and that phenylpropionic and phenylacetic acids were products of putrefaction (Salkowski); phenylalanine was therefore regarded, as suggested by Tiemann, as the constituent from which these substances arose, but the actual presence of phenylalanine in the proteins was only proved when E. Fischer commenced his investigations upon the proteins. He then found that in some proteins it exceeded in amount that of tyrosine, and that it was in fact the principal aromatic constituent. Those proteins, such as gelatin, in which its presence was demonstrated by Spiro, and which contains no tyrosine, was found to contain phenylalanine as its aromatic constituent.

The constitution of phenylalanine was determined in 1882 by Erlenmeyer and Lipp, who synthesised it by Strecker's method from phenylacetaldehyde, hydrogen cyanide and ammonia:—

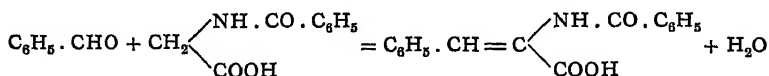


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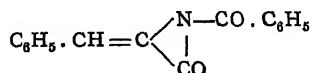
This synthetical substance closely resembled Schulze and Barbieri's natural compound, and their identity was established. Posen's preparation, described under this name, was at the same time shown to be phenyl- β -aminopropionic acid.

In 1893 a new method of synthesising amino acids, starting from hippuric acid, was introduced by Erlenmeyer jun., phenylalanine being the first product to be prepared.

When benzaldehyde is condensed with hippuric acid in the presence of acetic anhydride a Perkin's reaction takes place and benzoyl- α -amidocinnamic acid is formed :—

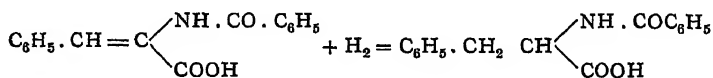


Under the influence of acetic anhydride, this is converted into the lactimide,

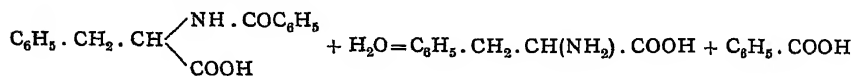


which by hydrolysis by acids or by alkalis is reconverted into benzoyl- α -amidocinnamic acid.

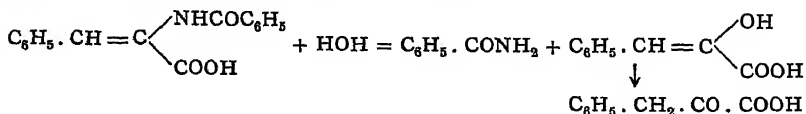
Benzoyl- α -amidocinnamic acid is reduced by sodium amalgam or by zinc and hydrochloric acid to benzoyl- α -amino- β -phenyl-propionic acid :—



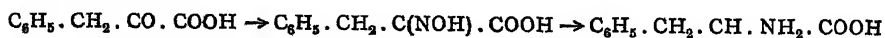
from which the benzoyl group is easily removed by hydrolysis with the formation of phenylalanine :—



Benzoyl- α -amidocinnamic acid is converted by the action of acids or alkalis into phenylpyruvic acid and benzamide,

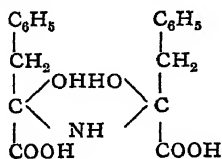


which Erlenmeyer proved by preparing the oxime and reducing it to phenylalanine :—

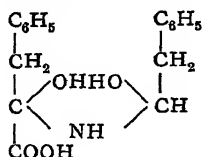


Benzoyl- α -amidocinnamic acid is also converted by the action of ammonia into a compound, which yields phenylalanine on hydrolysis.

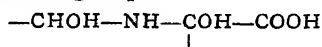
The mechanism of this reaction was explained by Erlenmeyer and Kunlin in 1899. Just as benzoyl- α -amidocinnamic acid is converted by alkali into phenylpyruvic acid and benzamide, so also does this reaction take place with ammonia; the phenylpyruvic acid then reacts with ammonia giving a body of the composition,



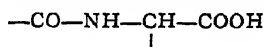
which loses carbonic acid yielding,



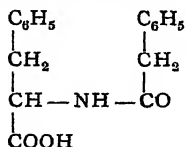
This substance contains the group,



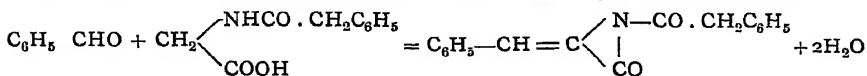
which, by rearrangement and by the loss of a molecule of water, becomes



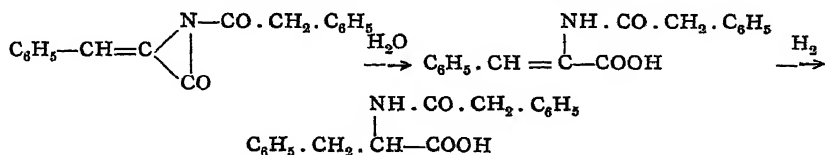
and it therefore changes into phenylacetyl-phenylalanine:—



By subsequent hydrolysis phenylalanine and phenylacetic acid result. The proof of this reaction was given by the synthesis of phenylacetyl-phenylalanine by condensing benzaldehyde with phenaceturic acid.—



hydrolysing the resulting lactimide, just as in the case of benzoyl- α -amidocinnamic acid, reducing it with sodium amalgam:—



and showing the identity of the two substances.

Tyrosine.

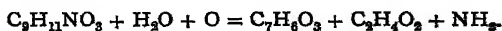
By fusing cheese with caustic potash Liebig, in 1846, obtained a new compound, consisting of a mass of fine silky needles, soluble with difficulty in water; he named it tyrosine from *τυρος*, cheese. The same substance was isolated by Warren de la Rue from cochénille, and a year later, in 1849, Hinterberger obtained it by the hydrolysis of horn. Its presence in albumin, fibrin and caseinogen was demonstrated by Bopp.

The results of numerous investigations were published in 1860 by Stadelér, who found tyrosine in silk-fibroin, mucin and various other proteins, and who also noted its occurrence in the free state in various organs, generally in conjunction with leucine. Since then, tyrosine has been constantly obtained from proteins by hydrolysis with acids and by the action of trypsin, and has long been regarded as a constituent of the protein molecule.

Its formula $C_9H_{11}NO_3$ was determined by Warren de la Rue and by Hinterberger. Strecker, in 1850, showed that it behaved like leucine and glycine, but pointed out that it did not belong to this series; and Wicke, in 1857, suggested that it stood in the same relation to the series of aromatic acids as glycine and leucine did to the fatty acids. Stadelér was really the first to show that tyrosine was an aromatic compound, when he obtained chloranil (tetrachloroquinone) from it by the action of chlorine; he also found that it had a constitution similar to that of glycine and leucine. Fröhde also held this view, but Thudichum and Wanklyn, as they could not obtain picric acid from tyrosine by the action of nitric acid, considered that it was not an aromatic compound.

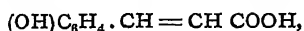
Stadelér's discovery of the formation of chloranil from tyrosine led to the supposition that tyrosine was a derivative of salicylic acid, and on this assumption Schmidt and Nasse attempted to synthesise tyrosine from ethylamine and iodosalicylic acid, and from amidosalicylic and ethyl iodide, but did not succeed. On heating tyrosine they obtained a base $C_8H_{11}NO_2$, which they thought analogous to the one Schmidt had obtained by heating amidosalicylic acid; on this account they held to the accuracy of the theory that tyrosine was ethylamidosalicylic acid.

A great advance was made by Barth in 1865, who showed that tyrosine was not ethylamidosalicylic acid. As yet salicylic acid had never been obtained from tyrosine, and Barth, in his attempt to prepare this compound from tyrosine by oxidation, by fusion with potash, obtained para-oxybenzoic acid and acetic acid, the decomposition taking place as follows:—



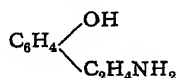
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He concluded that tyrosine was related to paracumaric acid,

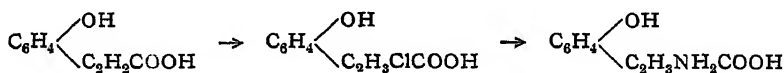


in the same way as alanine was related to acrylic acid. Ost confirmed this result of Barth's several years later, when he obtained p-oxybenzoic acid by fusing tyrosine with caustic soda.

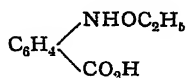
Tyrosine was now regarded as ethylamidopara-oxybenzoic acid; on reduction, therefore, it should yield ethylamine, but instead of this Hufner, in 1868, obtained ammonia, and he supposed tyrosine to be amidophloretic acid.¹ This view was strengthened when Barth in the following year obtained p-oxybenzoic acid from phloretic acid and also from Schmidt and Nasse's base. This here garded as



and tyrosine as oxyphenylamidopropionic acid, the nitrogen being attached to the side chain and not to the benzene ring as supposed by Schmidt and Nasse. Barth's attempt to synthesise tyrosine from paracumaric acid by the following reactions



which were also put forward by Beilstein and Kuhlberg, was not sufficiently successful to prove that tyrosine had this formula, so that Ladenburg, who stated that the reactions of tyrosine could be just as well explained by the formula



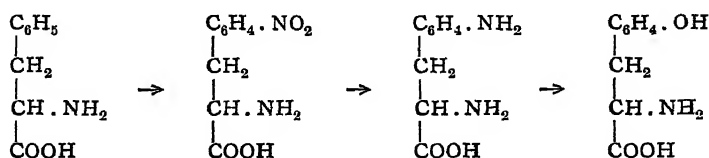
synthesised this compound. It was quite different to tyrosine, and Barth's formula was therefore correct.

The work of Baumann in 1879 upon the decomposition of tyrosine by putrefaction showed that hydroparacumaric acid or p-oxyphenylpropionic acid was the first product and that tyrosine must be p-oxyphenylaminopropionic acid. It only remained to determine the position of the NH_2 group, whether it was in the α - or β -position.

This was decided in 1882 by Erlenmeyer and Lipp who synthesised tyrosine from phenylalanine. Their first method to prepare p-sulpho-

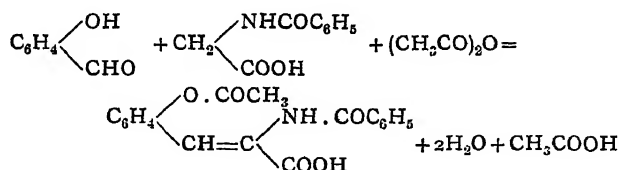
Until 1900 phloretic acid had the constitution $\text{OH} \cdot \text{C}_6\text{H}_4 \cdot \text{CH} \cdot \text{COOH}$, but in that year Bougault showed it to be β -oxyphenylpropionic acid $\text{OH} \cdot \text{C}_6\text{H}_4 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$.

phenyl- α -aminopropionic acid and to exchange the sulpho-group for the hydroxyl group was not successful, as in the fusion with potash the side chain also became oxidised and no tyrosine resulted. They then prepared p-nitrophenylalanine, and converted it into p-amidophenylalanine; on treating this latter compound with the calculated quantity of sodium nitrite and warming, they obtained p-oxyphenylalanine, thus,

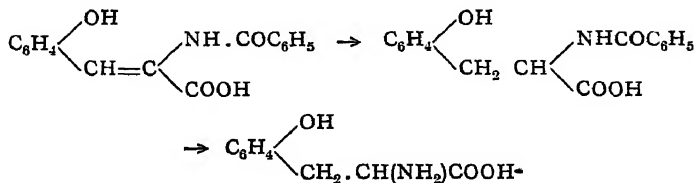


This compound had the same properties as the natural tyrosine, which was thus proved to be p-oxyphenyl- α -aminopropionic acid.

Erlenmeyer jun., and Halsey, in 1899, synthesised tyrosine by the condensation of hippuric acid with p-oxybenzaldehyde in the presence of acetic anhydride. The reactions are the same as those described by Erlenmeyer for the synthesis of phenylalanine, except that the hydroxyl group of the p-oxybenzaldehyde becomes acetylated in the process:—



The lactimide is again formed, but, on hydrolysis by alkali, the acetyl group is removed and p-oxy- α -benzoylaminocinnamic acid is obtained. On reduction it yields benzoyltyrosine, from which tyrosine is formed by hydrolysis:—

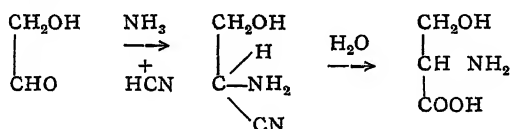


Just as in the case of phenylalanine, p-oxy- α -benzoylaminocinnamic acid when treated with ammonia yields an α -oxo acid, which reacts with ammonia, giving a complex compound; this, on hydrolysis, by heating in a sealed tube with hydrochloric acid, is converted into tyrosine.

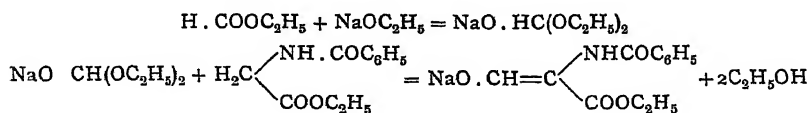
Serine.

Serine is, as yet, the only member of the oxyamino acids of the aliphatic series which has been isolated with certainty from the mixture of decomposition products of the proteins. It was first obtained in 1865 by Cramer from silk-gelatin, and was not again obtained until E. Fischer isolated it from the various proteins which he and his pupils have examined.

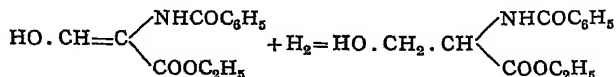
Cramer, the discoverer, showed that, when serine was treated with nitrous acid, it was converted into glyceric acid, and he recognised it as an aminolactic acid. It was regarded as α -amino- β -oxypropionic acid, but this was only definitely proved when it was synthesised by Fischer and Leuchs in 1902 from glycollic aldehyde, hydrogen cyanide and ammonia, which is the first instance of the employment of Strecker's method to build up oxyamino acids from oxyaldehydes.



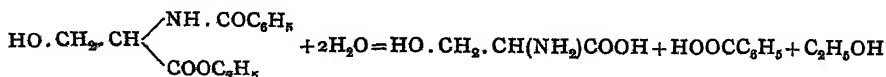
Serine is another of the amino acids which Erlenmeyer jun. synthesised in 1902 from hippuric acid, and which he described in detail with Storp in 1904; by condensing formic ester and hippuric ester with sodium ethylate they obtained oxymethylene hippuric ester or formyl hippuric ester.—



The free ester, obtained from the sodium salt as a thick oil, on reduction with aluminium amalgam gave N-benzoyl serine ester:—

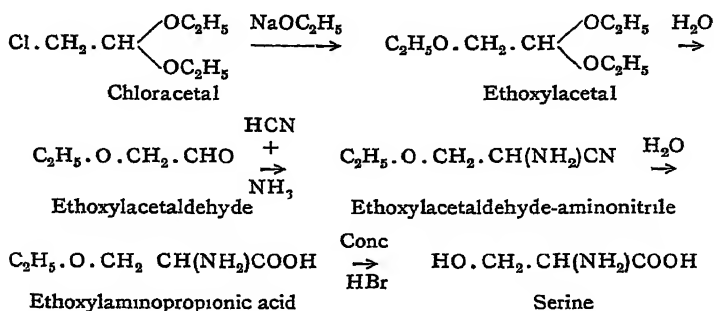


which, when hydrolysed with dilute sulphuric acid, was converted into benzoic acid and serine:—



A better method of synthesising serine was described by Leuchs

and Geiger in 1906, and was carried out as follows, starting from chloroacetal :—



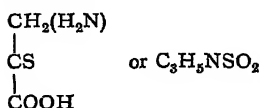
Cystine.

Under the name of cystic oxide, a new species of urinary calculus, this compound was first described by Wollaston in 1810. Lassaigne found it under the same conditions in a dog in 1823. Its presence in the kidney of an ox was shown by Cloetta in 1856, and in the following year Scherer found it in the liver of a patient, who had died of typhoid fever. The name cystine was given to it by Berzelius. Drechsel, in 1891, isolated it from horse's liver and in 1896 from a porpoise, and then first regarded it as a normal product of metabolism. In 1890 Kulz obtained cystine by the digestion of fibrin with pancreas, and Emmerling, in 1894, found it mixed with tyrosine which he had prepared by the hydrolysis of horn. An attempt was made by Suter, in 1895, to obtain it from horn, but he could only obtain α -thiolactic acid, and not until 1899 was it shown by K. A. H. Mörner to be a product of hydrolysis of this protein, and, in 1901, of other proteins also. His results were confirmed by Embden, who was working independently, and who also obtained cysteine, which is derived from cystine as proved by Patten

The earliest analyses of cystine are given by Prout, who overlooked the fact that cystine contained sulphur, the presence of which element was first shown by Baudrimont and Malagutis. Thaulow gave cystine the formula $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}$, and pointed out that it was one of the few compounds made up of five elements. On account of the uneven number of atoms in its molecule, Gmelin replaced this formula by $\text{C}_3\text{H}_7\text{NSO}_2$, which formula was confirmed by Grote in 1864, and later by Külz in 1884.

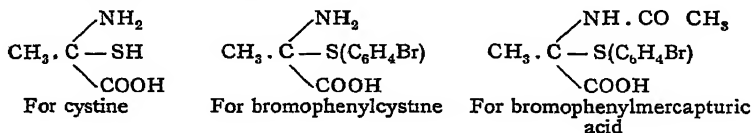
The first investigations on the constitution of cystine are those of Dewar and Gamgee in 1871, who, on treating cystine with nitrous acid, obtained an acid which they thought was pyruvic acid, $\text{CH}_3 \cdot \text{CO} \cdot \text{COOH}$,

and on this account gave cystine the constitution of

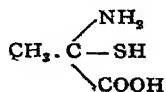


Hoppe-Seyler, as cited by Baumann and Preusse, showed that the nitrogen of cystine was separated off as ammonia by alkalis and not as methylamine, as would be expected from this formula, and moreover maintained that the formula was $\text{C}_3\text{H}_7\text{NSO}_2$.

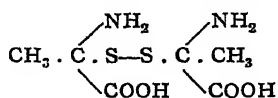
Baumann and Preusse's investigations in 1881 upon the fate of bromobenzene in the animal body, though they were only indirect evidence in regard to the constitution of cystine, were of great importance, as they were carried out at the time when cystine was a very scarce compound and only obtainable from calculi. They found that, when bromobenzene was given to animals, it was excreted in the urine in combination with a sulphur-containing compound, which combination had the formula $\text{C}_{11}\text{H}_{12}\text{BrSNO}_3$, and in this they were confirmed by Jaffé. When boiled with hydrochloric acid, this compound was converted into acetic acid and a substance $\text{C}_9\text{H}_{10}\text{BrNSO}_2$, from the empirical formula of which Baumann and Preusse supposed that it was cystine $\text{C}_3\text{H}_7\text{NSO}_2$, in which one of the hydrogen atoms was replaced by $\text{C}_6\text{H}_4\text{Br}$. Their further experiments led them to the conclusion that it was really a derivative of cystine. On decomposition by alkali, this latter compound yielded bromophenylmercaptan, ammonia and another substance, which they eventually recognised must be pyruvic acid. It had been shown that Dewar and Gamgee's formula for cystine, which was also based upon the formation of pyruvic acid, was not accurate, so they proposed the following:—



Baumann next found that cystine, on reduction with zinc and hydrochloric acid, was converted into a new base, which he called cysteine; this gave the same products on decomposition as cystine, into which it was easily reconverted by oxidation. He therefore recognised that these compounds were related to each other, as a mercaptan is to a disulphide; consequently the formula



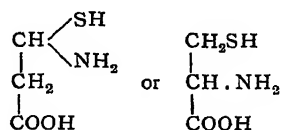
was really that of cysteine, and that of cystine was



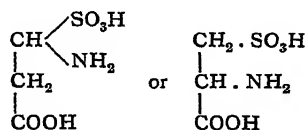
the former bromophenylcystine being bromophenylcystine, etc.

The actual formation of pyruvic acid from various mercapturic acids upon which these formulæ for cysteine and cystine were founded, was only shown later by Baumann's pupils, Königs, Brenzinger and Schmitz, and in conjunction with Suter's observation that α -thiolactic acid was formed by the hydrolysis of horn, this formula for cystine was accepted. The results obtained, however, scarcely justified this formula as pointed out by Friedmann in 1902, who showed conclusively that the cystine, obtained from proteins, had not this constitution.

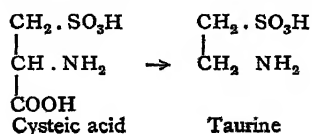
It had been found by Jochem in Hofmeister's laboratory that amino acids, when treated with nitrous acid in hydrochloric acid solution, were converted into the corresponding chloro-derivatives, and Friedmann, on applying this reaction to cystine obtained dichlorodithiopropionic acid; this when reduced gave β -thiopropionic acid, and on subsequent oxidation β -dithiopropionic acid, identical with the compound prepared from β -iodopropionic acid and potassium hydrogen sulphide. The sulphur atoms in cystine and cysteine were therefore in the β -position, and it remained to show in which position the amino group was situated, whether as



By oxidising cystine with bromine water, Friedmann obtained cysteic acid, *i.e.*, either



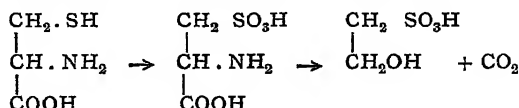
which, when heated, by loss of carbon dioxide, was converted into taurine, which is only explainable by the second formula



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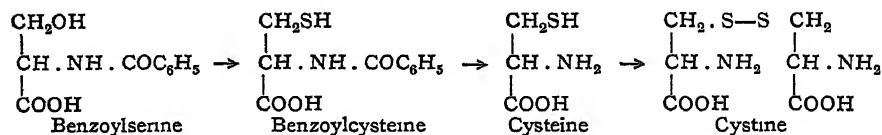
These reactions also showed how taurine might originate in the body from cystine.

At about the same time Neuberg, by treating cystine with nitric acid, obtained isethionic acid, which pointed to the correctness of Friedmann's formula, it at any rate showed that the sulphur and nitrogen atoms were attached to different carbon atoms. In the reaction the SH group was oxidised to the SO₃H group, and the NH₂ group was converted into the OH group by nitrous acid formed in the oxidation:—



The synthesis of cystine by Erlenmeyer jun. in 1903, which was more fully described by him and Storp in 1904, showed that Friedmann's formula was correct:—

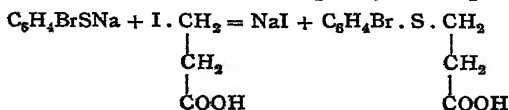
Benzoylserine was heated with phosphorus pentasulphide, and the product after hydrolysis with hydrochloric acid, gave cystine which was converted by oxidation into cystine:—



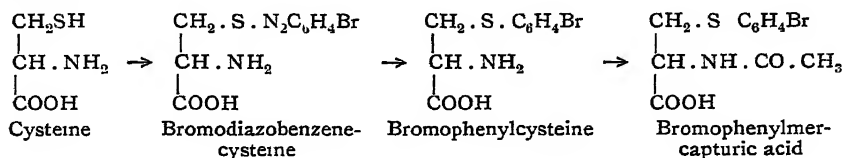
Another synthesis of cystine was described by Fischer and Raske in 1908 (see page 75) which is similar to that of Erlenmeyer.

The formation of bromophenylmercapturic acid from bromobenzene and cystine in the organism, if it had the formula given it by Baumann now seemed scarcely possible, unless an isomeric α -thio- β -aminopropionic acid were also present in the protein molecule together with the di- β -thio- α -aminopropionic acid or cystine. The investigation of their constitution was therefore taken up by Friedmann in 1904, who succeeded in showing that they were also derived from β -thio- α -aminopropionic acid and not from the isomeric α -thio- β -aminopropionic acid.

By the action of nitrous acid in hydrochloric acid solution on bromophenylcystine, prepared by Baumann's method, chlorobromophenylthiopropionic acid was obtained, which, on reduction, gave bromophenylthiolactic acid. This was identical with the substance prepared from β -iodopropionic acid and sodium bromophenylmercaptan:—



and therefore the SH group was in the β -position. Further proof was given by Friedmann by the synthesis of bromophenylmercapturic acid from cysteine. p-Bromodiazobenzene chloride was combined with cysteine; this compound, when decomposed by dilute soda, gave bromophenylcysteine, which, on acylation, was converted into bromophenylmercapturic acid:—



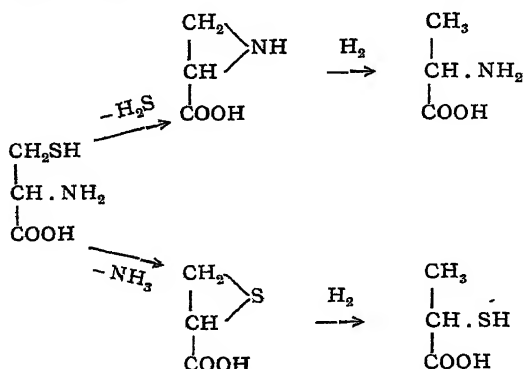
It was first observed by Suter, in 1895, that α -thiolactic acid was formed by the hydrolysis of proteins, and it was constantly obtained by Friedmann. It was always regarded as a secondary product, but its formation from cystine could not be explained, as cystine is a β -thiopropionic acid

In 1904 Mörner found that pyruvic acid was a constant product of hydrolysis of proteins, and that this compound gave α -thiolactic acid with hydrogen sulphide. Its formation was thus explained, but it was curious that hair, which is very rich in sulphur, gave less pyruvic acid than horn, which is less rich, and that caseinogen, which contains very little sulphur, also gave it. Mörner therefore supposed that there was another sulphur-containing compound in the protein molecule, which supposition was strengthened by Neuberg and Mayer's statement that stone cystine differed from protein cystine in many of its physical properties. Mörner's subsequent work on the decomposition of stone cystine, when he obtained α -thiolactic acid, ammonia and alanine helped to support this idea, he regarded the alanine as formed from cystine and the α -thiolactic acid from the isomeric α -thio- β -aminopropionic acid, both of which he supposed were present in the stone cystine in equal quantities.

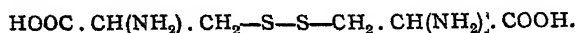
Fischer and Suzuki soon afterwards showed that Neuberg and Mayer's stone cystine contained tyrosine, and that its different behaviour to protein cystine was due to the presence of this compound. Rothera also could find no difference between stone cystine and protein cystine, and further, Gabriel's synthesis of isocysteine or α -thio- β -aminopropionic acid and isocystine, which had quite different properties to cystine, though the two were much alike in many of their reactions, proved that stone cystine and protein cystine must be identical substances. Finally, it has been shown by Friedmann that α -thiolactic acid, ammonia

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and alanine can be obtained from protein cystine, which decomposition may take place according to Gabriel in the following way :—



Thus, the work of Friedmann on the constitution of cystine, its synthesis by Erlenmeyer jun. and by Fischer, definitely show that it has the composition



The proof that bromophenylmercapturic acid is derived from cysteine, the formation of α -thiolactic acid from cystine derived either from stones or proteins, and the identity of protein cystine with stone cystine show that cystine is the only sulphur-containing compound in the protein molecule: and also that the number of sulphur atoms in the protein molecule is two or a multiple of two, instead of the variable number which had been determined by the earlier workers upon the sulphur in the protein molecule. This work was commenced by Mulder, who was the first to observe that albumin, caseinogen, etc., when heated with alkali gave off hydrogen sulphide; in consequence of this he regarded these compounds as composed of sulphur and protein in various proportions. Fleitmann, a pupil of Liebig's, in 1847 then showed that this view of the constitution of albumin, etc., was erroneous, for he found that only a portion of the sulphur was split off by alkali, and that a portion still remained combined with the protein. The later investigators upon this question—Nasse, Danilewsky, Kruger, Suter, Malerba, Schulz—confirmed Fleitmann's results, and in addition they determined the ratio of total sulphur to loosely bound sulphur, as this sulphur easily split off by alkali was called. Their results varied considerably, and this was due to the different methods which they employed. In some proteins, *e.g.*, serumalbumin, the ratio of loosely bound sulphur to total sulphur was as 2 : 3, in others 1 : 2 or 5 : 3. From these values determinations were made of the molecular weight: thus serumalbumin was given a mole-

cular weight of 5,100, egg-albumin of 4,900, globulin of 4,600, edestin of 7,300. Mörner's isolation of cystine from proteins, which he found also lost only a portion of its sulphur—about 75 per cent.—when boiled with alkali, did not at once prove that cystine was the only sulphur-containing compound in the molecule of all proteins; this was really only proved by Friedmann's work.

B. MONOAMINODICARBOXYLIC ACIDS.

Aspartic Acid.

Asparagine, the amide of aspartic acid, was first isolated by Robiquet and Vauquelin, in 1806, from the juice of *Asparagus officinalis*; hence its name. Not only is asparagine found in asparagus, but also in the seedlings of lupines, peas, vetches, etc., from which it is best and most easily prepared.

Aspartic acid was first obtained by Plisson, in 1827, from asparagine by boiling it with lead hydroxide, and is usually prepared from this compound by hydrolysis with alkali or acid.

Only however in 1868 was the presence of aspartic acid in vegetable proteins shown by Ritthausen, who obtained it by the hydrolysis of conglutin and legumin with sulphuric acid; in the following year Kreussler obtained it in the same way from animal proteins. In 1874 Radziejewski and Salkowski found that it was a product of the tryptic digestion of proteins, and the asparagine in plants most probably arises from the aspartic acid of the protein in the seed.

Its composition, $C_4H_7NO_4$, was established in 1833 by Boutron-Charlard and Pelouze, and confirmed by Liebig. In 1848 Piria showed that aspartic acid was converted into malic acid by the action of nitrous acid, and he regarded aspartic acid and asparagine as the two amides of malic acid



corresponding to oxamic acid and oxamide.

This idea of their constitution was proved to be erroneous by Kolbe in 1862, who showed that aspartic acid did not give off ammonia when boiled with dilute caustic alkali, and that asparagine only lost half of its nitrogen when thus treated. Aspartic acid was therefore not the amide of malic acid, but amino-succinic acid, and asparagine the amide of this compound.

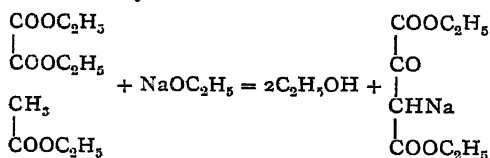
The first synthesis of aspartic acid is that by Dessaignes in 1850, who obtained a crystalline substance by heating acid ammonium malate to 160-200° C., which, when treated with hydrochloric or nitric acid, was

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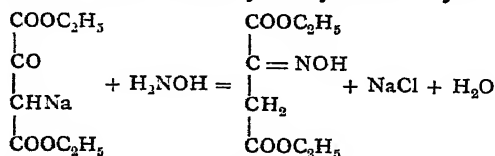
converted into aspartic acid. In the same way Dessaignes obtained aspartic acid from acid ammonium fumarate and maleate. At Liebig's suggestion these reactions were confirmed by Wolff. It was shown by Engel, in 1887, that aspartic acid could be obtained directly by heating maleic or fumaric acid with alcoholic ammonia to 140-150° without the formation of the intermediate substance which is fumarimide, and to which the following constitutional formulæ have been given:—



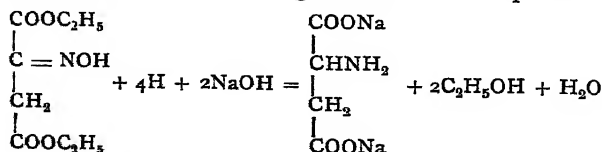
These syntheses give no indication as to the structure of aspartic acid, the constitutional formula of which is based upon Kolbe's work, that it is amino-succinic acid; the only synthesis of aspartic acid which confirms this constitution appears to be that by Piutti in 1887. Sodium oxalacetic ester, prepared from oxalic ester and acetic ester in the presence of sodium ethylate:—



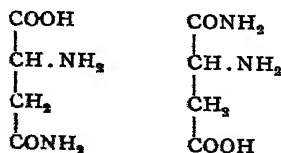
gives an oxime when treated with hydroxylamine hydrochloride:—



and this is reduced by sodium amalgam to sodium aspartate



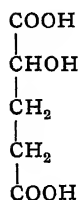
From this oxime Piutti has also prepared the two isomeric asparagines:—



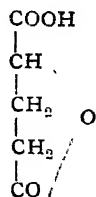
Glutamic Acid.

The presence of this amino acid in the protein molecule was shown by Ritthausen in 1866, who obtained it from wheat gluten by hydrolysis with sulphuric acid. He showed that it was an amino acid and termed it glutaminic acid, on account of its first preparation from gluten. Subsequently Ritthausen and Kreussler isolated it from the hydrolysis products of other vegetable proteins. Kreussler could not demonstrate its presence in animal proteins, in which it was afterwards shown to occur in 1873 by Hlasiwetz and Habermann. Not only is glutamic acid formed by acid hydrolysis, but also by the action of enzymes: Knieriem and Kutscher obtained it by the tryptic digestion of fibrin, and its amide, glutamine, is found in the extracts of seedlings as shown by Schulze, v. Gorup-Besanez and Scheibler.

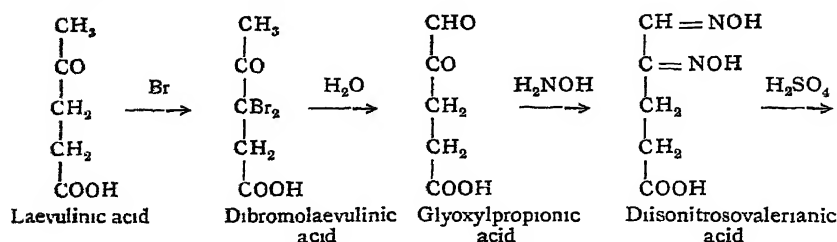
Ritthausen gave glutamic acid the empirical formula $C_5H_9NO_4$, and found that, when treated with nitrous acid, it was converted into an oxy-acid, which he termed glutanic acid and regarded as a homologue of malic acid. Dittmar again prepared glutanic acid and reduced it with hydriodic acid to an acid which was shown by Markownikoff to be what we now call glutaric acid, this was identical with the substance obtained by the hydrolysis of trimethylene cyanide, which was prepared from trimethylene bromide $CH_2Br.CH_2.CH_2Br$ and potassium cyanide. Glutanic acid differed from Simpson's β -hydroxyglutaric acid, and Markownikoff regarded it as the α -hydroxyglutaric acid,



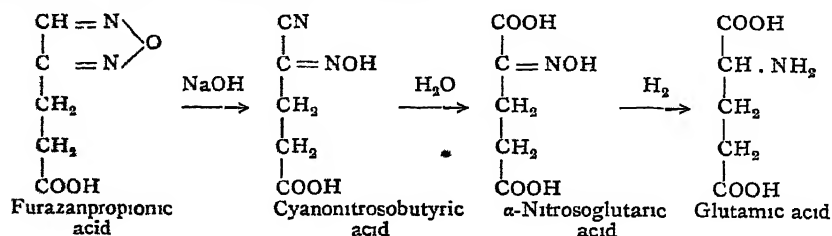
which, according to Bredt, exists in the free state as the γ -lactone.



Glutamic acid would therefore be α -aminoglutaric acid. The proof for this constitution was only given in 1890 by L. Wolff who synthesised glutamic acid from laevulinic acid. Dibromolaevulinic acid is obtained by bromination, and this when boiled with water gives diacetyl and glyoxylpropionic acid; diisonitrosovalerianic acid is formed from the latter, on treatment with hydroxylamine:

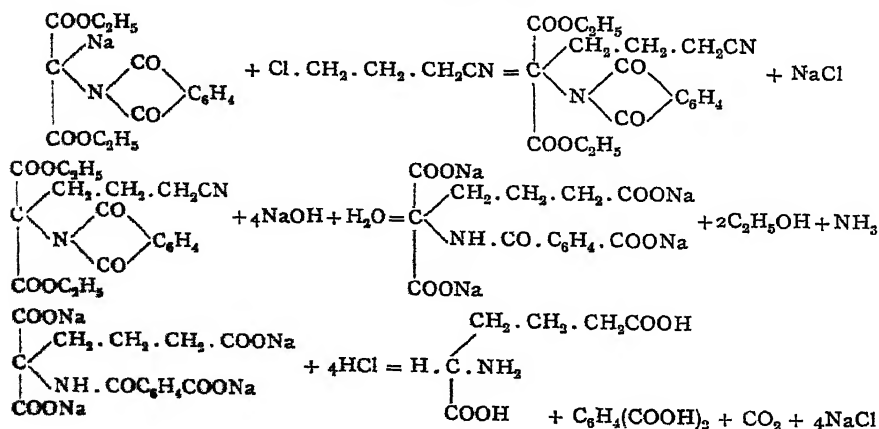


This is converted into furazanpropionic acid by sulphuric acid and then into cyanonitrosobutyric acid by caustic soda. Saponification changes the cyanonitrosobutyric acid into α -nitrosoglutaric acid from which glutamic acid is obtained by reduction:



This appears to be the only recorded synthesis of glutamic acid.

The next member of this homologous series, α -amino-adipic acid, has been prepared by Sørensen from phthalimidodisodium malonic ester and chlorobutyronitrile in the following way:—



Sørensen suggests that the same reactions might be employed for the synthesis of aspartic acid and of glutamic acid, in the case of the former condensing the sodium phthalimidomalonic ester with chloroacetic ester and in that of the latter with β -chloropropionic ester

$\text{Cl} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOC}_2\text{H}_5$. In view of our having only one synthesis of each of these two amino acids, and these syntheses being somewhat arduous, Sørensen's suggestion might with advantage be carried out.

C. DIAMINOMONOCARBOXYLIC ACIDS.

Drechsel's discovery of lysine amongst the products of hydrolysis of caseinogen in 1889 first showed that the monoamino acids were not the only constituents of the protein molecule; the substance, lysatinine, which he and his pupils also isolated a few years later from several proteins, was shown by Hedin to be a mixture of arginine and lysine, the former body having been many years previously obtained by E. Schulze and E. Steiger from germinating seedlings. Though ornithine had been discovered over ten years before lysine, its importance as a constituent of the protein molecule was not recognised until it was shown by Schulze to be a constituent of arginine. Histidine, discovered in 1896 by Kossel, was classed with the diamino acids until its constitution was determined, on account of its method of separation and its close relationship in many of its properties to arginine and lysine, the three bases having been termed by Kossel as the hexone bases and regarded as a very important portion of the protein molecule.

The synthesis of the diamino acids, in comparison with that of the monoamino acids, is very much more difficult and has only been achieved within the last few years.

Diamino-acetic Acid.—This acid, the first member of the series, was described by Drechsel as a decomposition product of caseinogen. Its existence is extremely doubtful, its attempted synthesis by Klebs did not succeed, and Willstätter could only obtain certain of its derivatives.

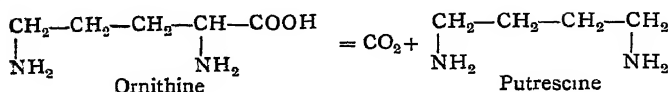
Diaminopropionic Acid has not yet been described as a constituent of the proteins, but it was synthesised by Klebs in 1894 by the action of ammonia upon dibromopropionic acid.

Diaminobutyric Acid.— α - γ -Diaminobutyric acid was prepared in 1901 by E. Fischer by the same method as he employed in the synthesis of ornithine.

Ornithine or α , δ -diaminovalerianic Acid.—In 1877 Jaffé obtained from the urine of birds, which he had fed with benzoic acid, dibenzoyl ornithine or ornithuric acid, and from this substance he prepared ornithine chloride. He regarded it as a diaminovalerianic acid, the first known representative of the series of diamino acids, but only in 1898 was the position of the two amino groups definitely determined by Ellinger, who obtained putrescine from it by putrefaction; the identity of putrescine with tetramethylenediamine had been previously shown by

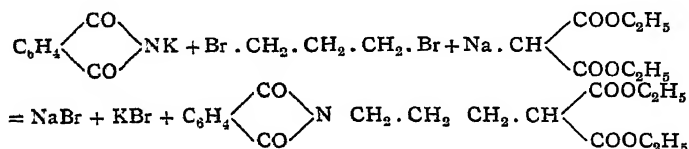
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Udransky and Baumann, and ornithine was therefore α , δ -diaminovalerianic acid, the hydrolysis of ornithine taking place according to the equation:—

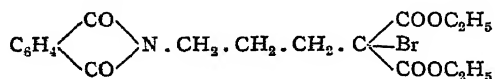


The expected synthesis of α - δ -diaminovalerianic acid, which was attempted by Willstätter in 1900, by the action of ammonia upon α - δ -dibromovalerianic acid, led to the synthesis of α -pyrrolidine-carboxylic acid, and only in the following year was the synthesis of this important naturally occurring diamino acid accomplished by E. Fischer. He made use of Gabriel's phthalimide method with a slight modification and obtained ornithine by the following series of reactions:—

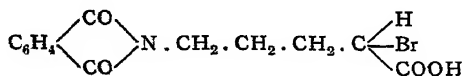
γ -phthalimidopropylmalonic ester was prepared from potassium phthalimide, propylene bromide and sodium malonic ester:



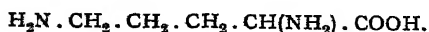
On bromination this gave phthalimidopropylbromomalonic ester,



which, on treatment with ammonia, did not give the desired result. On hydrolysis, however, and by loss of carbon dioxide, it is converted into δ -phthalimido- α -bromovalerianic acid,



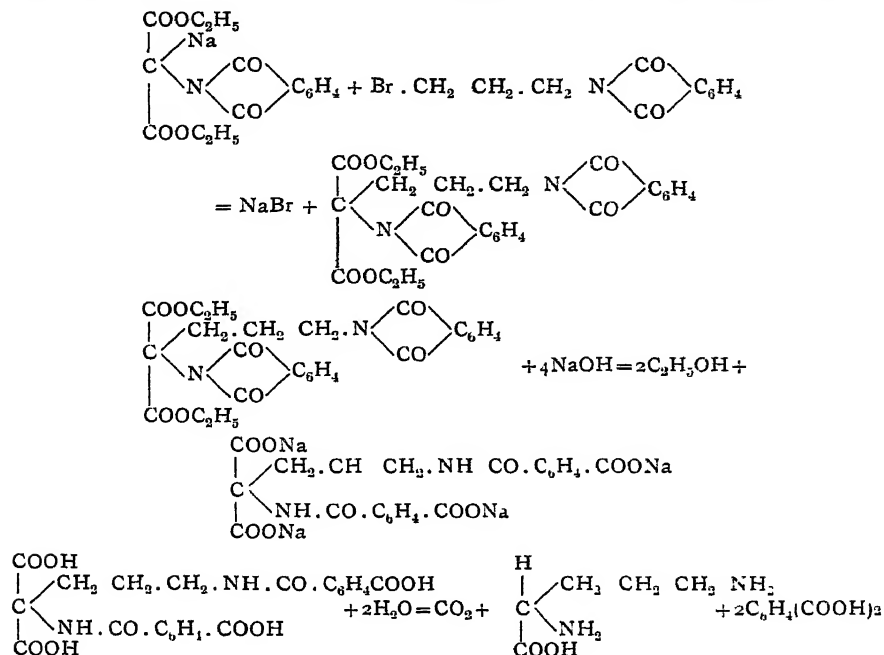
On treatment with ammonia, whereby Br is exchanged for NH_2 , and on subsequent hydrolysis, this acid yielded α - δ -diaminovalerianic acid or ornithine,



The dibenzoyl compound only differed from Jaffé's ornithuric acid by being optically inactive.

By a very similar series of reactions Sørensen has also synthesised ornithine: he first introduces the phthalimido group into the sodium

malonic ester and then allows γ -bromopropylphthalimide to act upon this; the new substance is reduced with sodium and alcohol, and, on subsequent hydrolysis of the acid, whereby the phthalyl groups are removed, and by loss of carbon dioxide, it yields ornithine, thus:—



Arginine.

In 1886 E. Schulze and E. Steiger obtained a nitrogenous base from the extracts of the germinated cotyledons of *Lupinus*, which had the composition $\text{C}_8\text{H}_{14}\text{N}_4\text{O}_2$, and to which they gave the name arginine; it was also found in the seedlings of other plants and is contained in all the vegetable proteins.

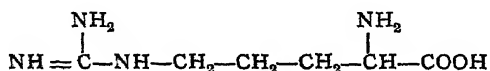
Hedin, in 1894, isolated it from the products of hydrolysis of horn, gelatin, conglutin, vitellin, egg-albumin, blood-serum, caseinogen. He also showed that Drechsel's lysatinine consisted of a mixture of arginine and lysine. From elastin both Bergh and Hedin failed to isolate it, but its presence in this protein was demonstrated by Kossel and Kutscher. Its occurrence in the protamines was shown by Kossel in 1896, and in histone from leucocytes by Lawrow in 1899. About the same time Kutscher found that it was contained in antipeptone, obtained by the tryptic digestion of fibrin; it is also formed when protamines are digested by trypsin (Kossel and Matthews).

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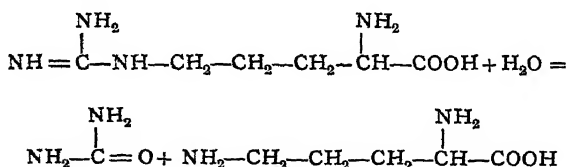
The identity of the arginine obtained from animal proteins with that from vegetable proteins was at first denied by Gulewitsch, but a little later he showed that they were identical, as also did Schulze.

The arginine, as it occurs in the proteins, is the dextro-rotatory modification except in fibrin, from which both Kutscher and Cathcart have isolated the inactive form.

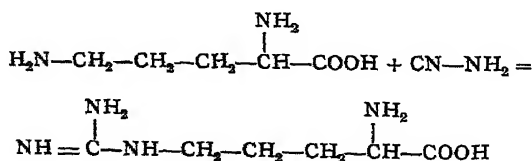
Schulze and Likiernik, in 1891, found that urea was formed when arginine was heated with baryta; they therefore supposed that arginine was a derivative of guanidine. In 1897 Schulze and Winterstein found that ornithine was also formed; they isolated it as its dibenzoyl derivative, which was found to be identical with Jaffé's ornithuric acid. Ornithine was regarded as a diaminovalerianic acid; Schulze and Winterstein showed that it contained two NH_2 groups not attached to neighbouring carbon atoms, and suggested that arginine, as it was a derivative of guanidine and ornithine, might have the following constitution:—



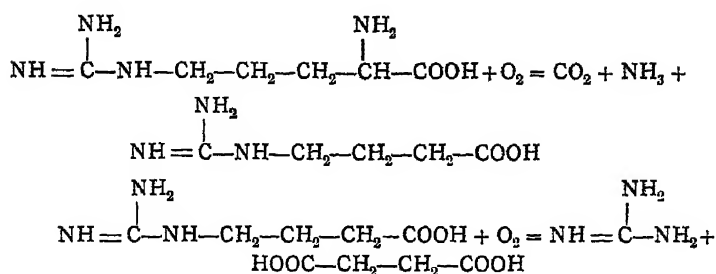
and that the formation of urea and ornithine might be explained according to the equation:—



Schulze and Winterstein, in 1899, proved that arginine was δ -guanidine- α -aminovalerianic acid by synthesis from cyanamide and ornithine:—



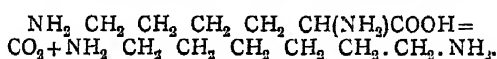
The presence of a guanidine group in arginine is also proved by the formation of guanidinebutyric acid and of guanidine and succinic acid by oxidation with permanganate, which probably takes place according to the following equations (Kutscher):—



Lysine.

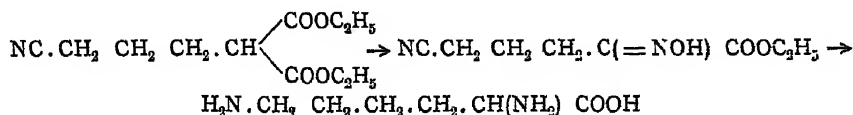
Lysine was discovered by E. Drechsel amongst the decomposition products of caseinogen in 1889, and its presence in other proteins—gelatin, egg-albumin, conglutin, fibrin—was shown by his pupils, Ernst Fischer, Siegfried and Hedin. It was found by Kutscher in antipeptone and by Kossel in the protamines. Its occurrence in germinating seedlings was demonstrated by Schulze and in vegetable proteins by Schulze and Winterstein. Thus, like arginine and histidine it is a very widely occurring constituent of the proteins

Drechsel gave it the formula $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2$ and regarded it as a diaminocaproic acid; Ellinger proved in 1899 that it possessed this constitution, by obtaining cadaverine from it by putrefaction, which showed that the two amino groups were in the α , ϵ -positions.—



Henderson's experiments also showed that lysine must have this constitution, namely, α , ϵ -diaminocaproic acid. Its constitution was only definitely determined by synthesis by Fischer and Weigert by the following method:—

When γ -cyanopropylmalonic ester is treated with nitrous acid, it loses one of its carboxethyl groups and is converted into α -oximido- δ -cyanovalerianic acid, which on reduction with sodium amalgam yields α , ϵ -diaminocaproic acid, thus:—

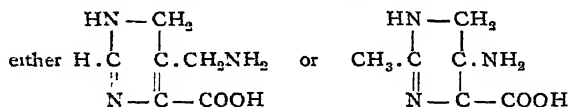


D. HETEROCYCLIC COMPOUNDS.

Histidine.

Histidine was discovered in 1896 by Kossel amongst the decomposition products of sturine, the protamine obtained from the ripe testis of the sturgeon. In the same year Hedin isolated a base from the products of hydrolysis of various proteins, which he regarded as identical with Kossel's histidine, and this was subsequently shown to be the case by Kossel and Kutscher. Kutscher also found it in antipeptone obtained by the pancreatic digestion of fibrin, and Schulze and Winterstein have shown that it occurs as a decomposition product of various vegetable proteins.

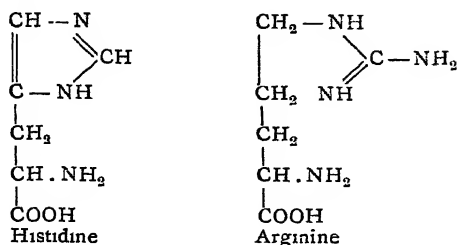
Histidine was found to possess the formula $C_6H_9N_3O_2$, but beyond the facts that it formed a dichloride, that two of its hydrogen were replaceable by metals and that it was optically active and therefore contained an asymmetric carbon atom, no experiments to determine its constitution were published until 1903. Herzog then showed that it gave the biuret reaction on warming, that it did not contain a methyl nor a methoxyl group, and that it was very resistant to oxidising reagents and in fact behaved as a saturated compound. At the same time Fränkel showed that it contained a carboxyl group and an amino group which was replaced by the hydroxyl group by the action of nitrous acid; it was therefore $(NH_2).C_5H_6N_2.CO_2H$. As it gave Weidel's pyrimidine reaction and did not contain a pyrrol ring nor a guanidine group, Fränkel suggested that it might be a derivative of dihydropyrimidine,



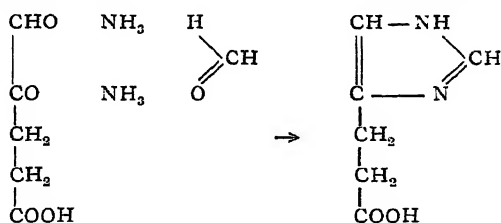
but Weigert pointed out that neither of these formulæ possessed an asymmetric carbon atom, and that histidine was optically active; consequently its formula must remain as $(NH_2).C_5H_6N_2.CO_2H$.

Pauly, in 1904, confirmed the presence of a carboxyl group, and showed that histidine contained a secondary amine group as well as a primary amine group by preparing a dinaphthalene sulpho derivative, the remaining nitrogen atom being probably a tertiary one. He pointed out that the resistance of histidine to oxidation and to acid permanganate, and that the formation of a di-silver compound were against the presence of a dihydropyrimidine ring in its molecule. These properties, as well as the capability which histidine possessed of forming azo-dyes with diazonium salts, pointed to the existence of a glyoxaline or imidazole ring in its composition. Pauly, therefore, gave it the constitu-

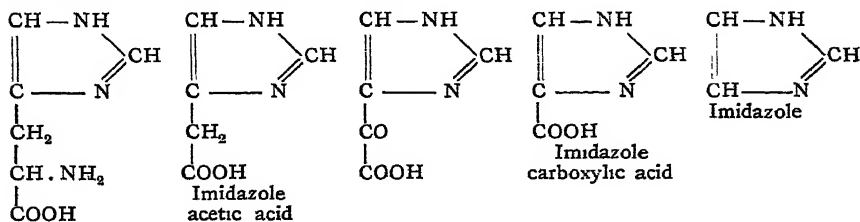
tion of imidazole-amino-propionic acid, at the same time showing its relation to arginine:—



This assumption of Pauly's was confirmed by Knoop and Windaus, who found that histidine is resistant to reduction by sodium and alcohol whereas the pyrimidine ring is very unstable towards this reagent. On reducing Fränkel's oxydesaminohistidine, which is obtained from histidine by the action of nitrous acid, they obtained β -imidazole-propionic acid.¹ This compound was identical with the synthetical product prepared from glyoxylpropionic acid, ammonia and formaldehyde.—



The presence of an imidazole ring in histidine was thus proved, and it only remained to show the position of the amino group. Fränkel urged certain objections against the presence of an imidazole ring in histidine, but Knoop and Windaus showed that these did not hold good. Knoop has since obtained imidazole-glyoxylic acid, imidazole-carboxylic acid, and imidazole from oxydesaminohistidine, and also imidazole-acetic acid. The imidazole ring is therefore in the β -position and histidine is β -imidazole- α -amino-propionic acid:



¹ Windaus and Vogt in 1908 showed that Fränkel's chlorohistidine carboxylic acid was the hydrochloride of β -imidazole-propionic acid.

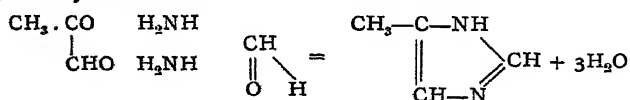
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In connection with histidine, the work of Windaus and Knoop on the formation of methylimidazole from glucose must be mentioned on account of the possible synthesis in the animal body of both histidine and purine bases.

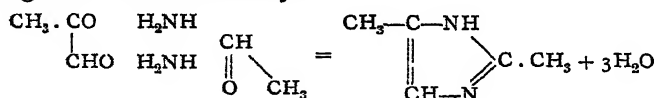
It has long been known that glucose is converted by alkalies into lactic acid and other oxy acids. Knoop and Windaus investigated this reaction in the presence of ammonia in the form of the strongly dissociated zinc hydroxide ammonia. By exposing glucose and zinc hydroxide ammonia to diffused daylight they obtained methylimidazole. From the sugar glyceric aldehyde is probably first formed, and this is converted into methyl glyoxal by loss of water, from which lactic acid may arise by the subsequent addition of water.



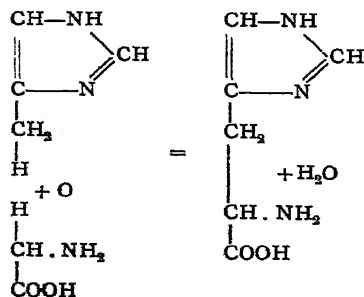
In the presence of ammonia and formaldehyde, also a product from the sugar, methylimidazole is formed as follows:—



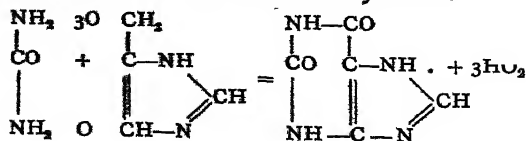
This condensation with formaldehyde as well as with methyl glyoxal is confirmed by the formation of dimethylimidazole when ammonia acts upon glucose and acetaldehyde.



From imidazole by condensation with glycocoll and simultaneous oxidation histidine may possibly be formed thus:—

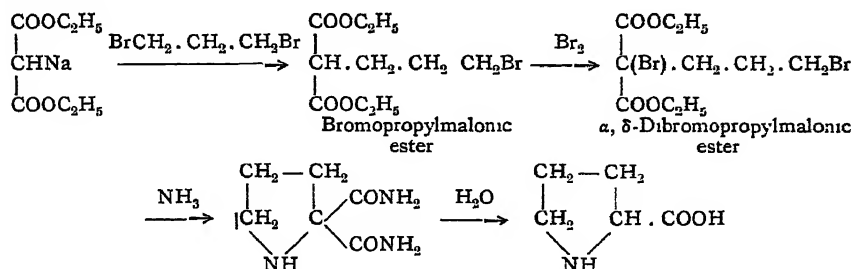


and by condensation with urea xanthine may arise:—

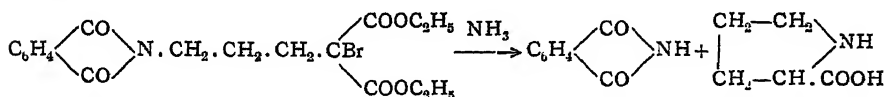


Proline.

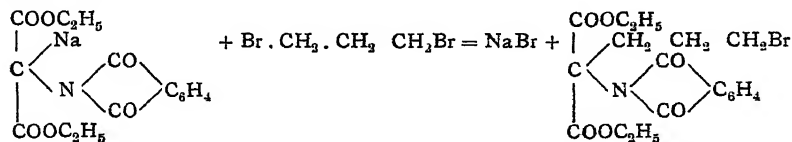
Just a year before E. Fischer obtained this compound by the hydrolysis of caseinogen, it was synthesised by Willstätter in 1900 from sodium malonic ester and trimethylene bromide by the following reactions:—



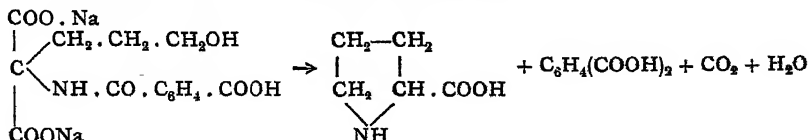
It was also synthesised by E. Fischer in 1901 from γ -phthalimidopropylmalonic ester which he employed in the preparation of ornithine. The bromine derivative of this compound when treated with ammonia gave a complex mixture of products which after hydrolysis by hydrochloric acid at 100° C. gave phthalimide and α -pyrrolidine carboxylic acid:—



Sørensen and Andersen in 1908 synthesised proline by the sodium phthalimidomalonic ester method, a yield of about 80 per cent. being obtained. Sodium phthalimidomalonic ester is condensed with trimethylene bromide,



the resulting γ -bromopropyl-phthalimidomalonic ester is heated in alcoholic solution with sodium hydroxide and the product so formed is evaporated with hydrochloric acid. Proline is obtained instead of the expected α -amino- δ -ethoxyvalerianic acid, ring formation occurring just as in the other methods of preparing proline:—



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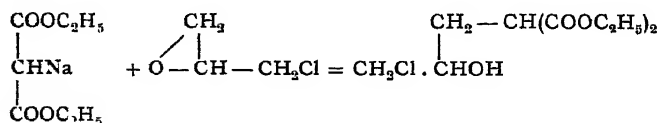
There was no difficulty in identifying the natural substance with the synthetical one, and its presence in egg-albumin, gelatin and other proteins was soon afterwards established.

The question at once arose whether this α -pyrrolidine carboxylic acid, or α -proline as Fischer termed it in 1904, was a primary product or a secondary product formed by the action of mineral acids upon other products, but its formation by hydrolysis by alkali and by the action of pepsin followed by trypsin decided that it was a primary product and therefore one of the units of the protein molecule. Sorensen, in 1905, suggested that it might arise from an α -amino- δ -oxyvalerianic acid which he synthesised, but the fact that this amino acid has not yet been obtained by hydrolysis of protein and the above facts seem to exclude this possibility.

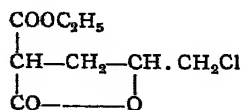
Oxyproline.

In 1902 E. Fischer isolated a compound of the empirical formula $C_5H_7O_3N$ from the hydrolysis products of gelatin. From its composition he supposed that it was an oxy-derivative of pyrrolidine carboxylic acid, and this was proved by its reduction to proline with hydriodic acid.

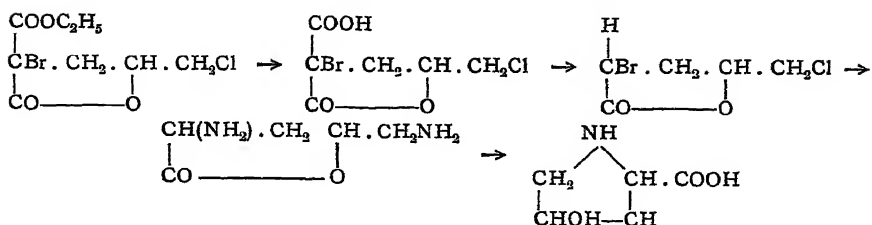
Leuchs, in 1905, synthesised two stereoisomeric- γ -oxy-prolines, one of which is probably the inactive form of the natural oxyproline. Epichlorhydrin and sodium malonic ester yield γ -chlor- β -oxy-propyl-malonic ester,



which loses alcohol and is converted into its lactone, *i.e.*, the ester of δ -chlor- γ -valerolactone- α -carboxylic acid,

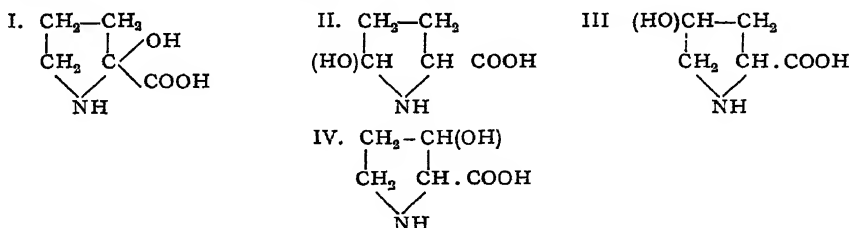


This compound on bromination, followed by hydrolysis of the ester group with hydrobromic acid and removal of carbon dioxide, gave α -brom- δ -chlor- γ -valerolactone, from which by treatment with ammonia γ -oxyproline was obtained:—



As this compound contains two asymmetric carbon atoms, four stereoisomeric forms are possible, by synthesis these must occur in two inactive forms. These forms Leuchs separated by crystallisation of the copper salts, the more insoluble acid being termed (*a*)- γ -oxyproline, the other (*b*)- γ -oxyproline.

The constitution of these acids was confirmed in 1908 by Leuchs and Felser, who converted them, by reduction with hydriodic acid, into proline. Their attempt to determine whether natural oxyproline was the active form of one of the synthetical compounds by converting the natural substance into its racemic form by heating with baryta to 200° C. was unsuccessful, since complete racemisation did not occur. As, however, all compounds containing one asymmetric carbon atom to which a carboxyl group is attached are easily racemised, the result led to the conclusion that oxyproline contains two asymmetric carbon atoms. Of the four possible formulæ,



formula I. is therefore excluded; and formula II. is not possible on account of the great stability of the acid to baryta; consequently the natural product can only be a γ - or a β -oxyproline.

Tryptophane.

The isolation of tryptophane by Hopkins and Cole in 1902 from the mixture of products formed by the tryptic digestion of caseinogen by precipitation in sulphuric acid solution with mercuric sulphate, besides adding to our list of foundation-stones or units of the protein molecule, gave us the explanation of three phenomena long known in connection with the chemistry of the proteins, namely (1) of the reddish-

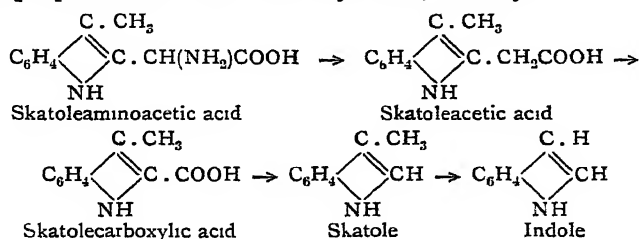
violet colour produced when chlorine or bromine water is added to a tryptic digest; (2) of the Adamkiewicz reaction; (3) of the origin of indole, skatole and related substances occurring in putrefaction.

The first of these phenomena was described in 1826 by Tiedemann and Gmelin. They observed that a reddish-violet colour was produced on adding chlorine water to an extract of dog's pancreas. Cl. Bernard, in 1856, showed that this reaction was given by a trypsin digest of caseinogen, and Kuhne, in 1875, found that bromine water gave a better reaction than chlorine water, whilst iodine did not produce the colour. Kühne showed also that this reaction was given by a pure trypsin digest in presence of chloroform, *i.e.*, without the intervention of micro-organisms, and was, in fact, the first to point out the difference between soluble ferments or enzymes, as he called them, and living ferments or bacteria. Stadelmann called the then unknown substance *proteinochromogen* and the coloured body *proteinochrome*, whereas Neumeister, who showed that the reaction was obtained with any deep-seated decomposition of protein, whether by trypsin, baryta water or dilute sulphuric acid gave the substance the name of *tryptophane*, which name Hopkins and Cole gave to their crystalline substance as it gave this reaction, and to whose presence in the digest the reaction is due.

Shortly before Hopkins and Cole isolated tryptophane, they studied the Adamkiewicz reaction—the production of a violet colour when concentrated sulphuric acid is added to a protein dissolved in glacial acetic acid—and found that it was caused by the presence of glyoxylic acid in the glacial acetic acid, from which it arose by the action of sunlight. On applying the glyoxylic reaction to tryptophane a very intense colour was produced, and hence the presence of tryptophane in the protein molecule is the cause of this reaction.

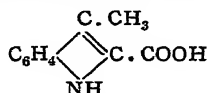
According to Cole, the Liebermann reaction—an intense blue colour when proteins are precipitated by alcohol and washed with ether and then heated with concentrated hydrochloric acid—is also due to the presence of tryptophane in the protein and to glyoxylic acid in the ether employed in washing the precipitated protein. The reddish-violet colour produced when proteins are heated with concentrated hydrochloric acid is due to tryptophane and to furfural formed from carbohydrate in the protein; it is very marked when cane sugar or furfural is added to a protein which does not give the reaction very strongly. Reichl's reaction again—a green to blue colour when proteins are heated with an aldehyde such as benzaldehyde, a drop of ferric chloride and concentrated hydrochloric acid—is due also to the presence of tryptophane in the protein.

The formation of indole by the putrefaction of proteins was observed by Kühne and by Nencki in 1874, that of skatole by Brieger in 1877, of skatolecarboxylic acid by E. and H. Salkowski in 1880, and of skatoleacetic acid by Nencki in 1889. Nencki regarded these substances as originating from a skatoleaminoacetic acid in the protein in a manner similar to that by which phenol, cresol, oxyphenylacetic acid and oxyphenylpropionic acid arose from tyrosine, namely:—

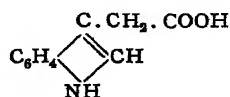


Tryptophane was found by Hopkins and Cole to have the empirical formula $\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}_2$ and to yield large amounts of indole and skatole when heated, and when subjected to putrefaction by bacteria the above-mentioned four products resulted. As under anaerobic conditions a large yield of skatoleacetic acid was obtained, and as skatole was the principal product when it was fused with potash, Hopkins and Cole regarded their substance as skatoleaminoacetic acid rather than the isomeric indoleaminopropionic acid.

The constitution of indole and skatole had been proved by synthesis, but that of the other two compounds had not been determined, and Nencki's formulæ for them were accepted. Investigations by Ellinger and Gentzen in 1903, who found that in the large intestine indole was formed in large amounts from tryptophane, but skatole only in small amounts, and that skatole only gave traces of indole under the same conditions led Ellinger to doubt the accuracy of Nencki's formulæ for skatoleacetic and skatolecarboxylic acids, more especially as Wislicenus and Arnold's skatolecarboxylic acid, which was synthesised from propionyl formic acid phenylhydrazine had the formula

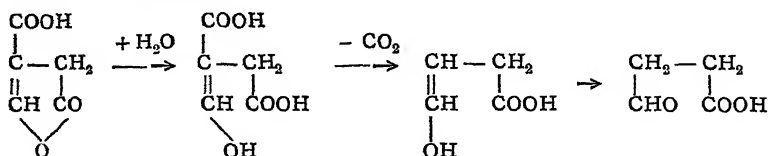


and was not identical with the putrefaction product, which might equally well possess the constitution of

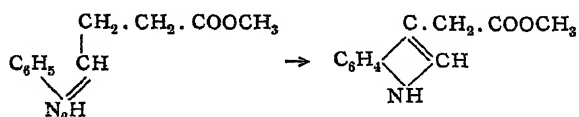


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The synthesis of this compound by Ellinger from β -aldehydopropionic acid, prepared from aconic acid as follows :—

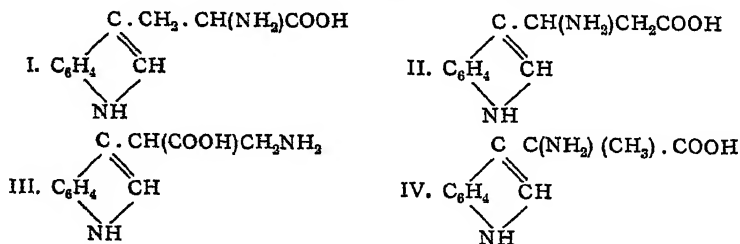


by condensation with phenylhydrazine, and the subsequent treatment of the ester of the hydrazone which was formed with alcoholic sulphuric acid,

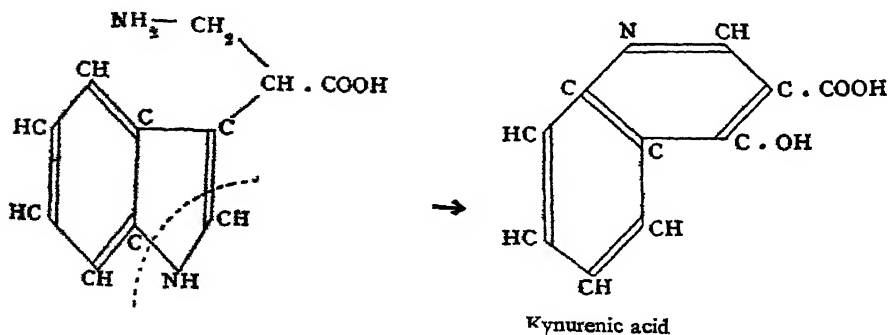


showed that it was identical with Salkowski's acid, and that it was indoleacetic acid and not skatolecarboxylic acid.

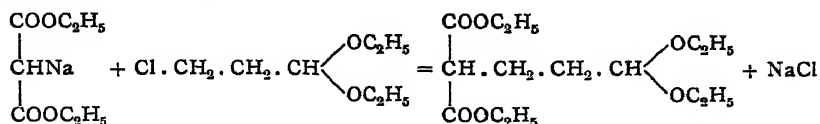
Four formulæ were therefore possible for tryptophane :—



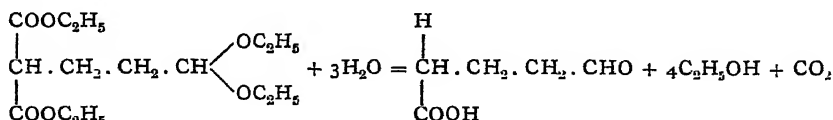
and as Ellinger had found that tryptophane when given to dogs and rabbits was converted into kynurenic acid, formula III. was regarded as the most probable for tryptophane, because it most easily explained this change :—



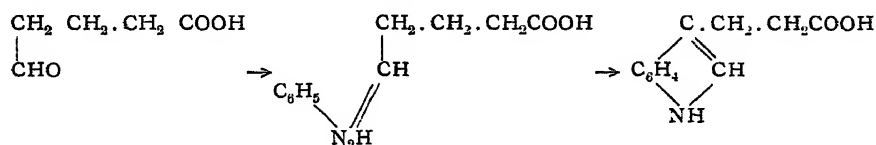
Ellinger's further work did not confirm this supposition. By condensing β -chloropropionacetal with sodium malonic ester he obtained propionacetal malonic ester,



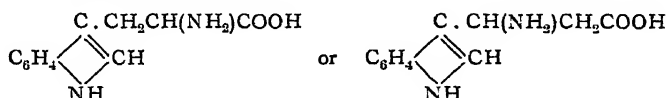
which, when heated in a sealed tube with water to 190° , was converted into γ -aldehydobutyric acid with loss of alcohol and carbon dioxide:—



The hydrazone of this compound when treated with alcoholic sulphuric acid gave the ester of indolepropionic acid:—

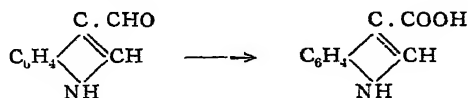


The indolepropionic acid obtained by hydrolysis was identical with Nencki's skatoleacetic acid, and tryptophane was therefore either



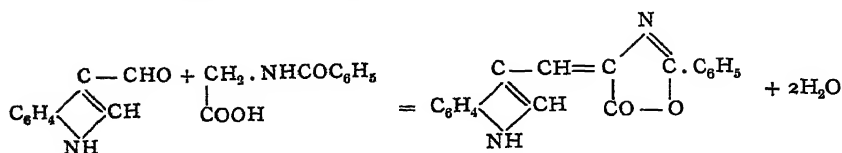
and kynurenic acid must be formed by some other reaction.

Hopkins and Cole had obtained by the oxidation of tryptophane with ferric chloride a body of the composition of $\text{C}_9\text{H}_7\text{NO}$, this body has been shown by Ellinger to be β -indole-aldehyde, firstly by oxidising it to β -indole-carboxylic acid,

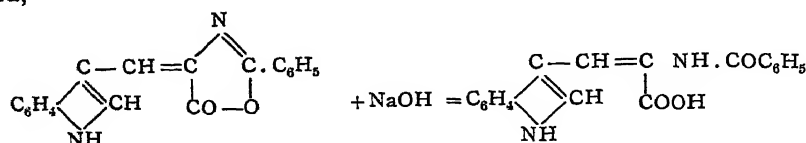


a compound synthesised by Ciamician and Zatti, and secondly by synthesis from indole and alcoholic chloroform. From this compound by the method employed by Erlenmeyer in the synthesis of phenylalanine, Ellinger and Flamand synthesised tryptophane in 1907.

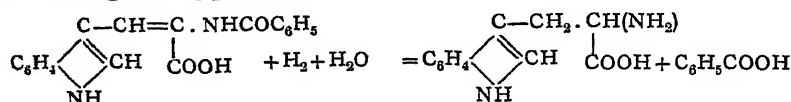
By condensing β -indolealdehyde with hippuric acid the azlactone is obtained,



which, when boiled with dilute soda, gives indolyl- α -benzoylaminoacrylic acid,



This compound on reduction with sodium amalgam and hydrolysis with water gives tryptophane:—



E. THE OPTICALLY ACTIVE AMINO ACIDS.

With the exception of glycine, all the amino acids contain an asymmetric carbon atom, and they are therefore capable of existence in two optically active forms. In one of these forms they are present in the protein molecule, and the synthesis of a naturally occurring amino acid is only completed when the synthesised compound has been separated into its optically active components.

Three methods are known, all due to Pasteur, by which an inactive mixture can be separated into its optically active isomers:—

1. The fractional crystallisation and mechanical separation of the two isomers.
2. The action of micro-organisms—moulds, yeasts—which destroy the one isomer more rapidly than the other. This is known as the biological method.
3. The fractional crystallisation of the salts of these compounds with optically active bases or acids.

By all these methods the optically active forms of the amino acids have been prepared.

By the first method Piutti, in 1887, obtained dextro- and lævo-aspartic acid. He fractionally crystallised natural asparagine and separated it into dextro- and lævo-asparagine, from which compounds he prepared both the dextro- and the lævo-aspartic acids. As, however, Piutti used asparagine from vetch seedlings, this separation cannot strictly be said

to be the actual synthesis of a natural compound, but there seems no reason to doubt the possibility of separating synthetical asparagine by this means.

Glutamic acid, the homologue of aspartic acid, according to Menozzi and Appiani, on recrystallisation from water, can be obtained in its two enantiomorphous forms.

By the second method Schulze and Bosshard prepared d-leucine and l-glutamic acid, and Engel prepared d-aspartic acid. Menozzi and Appiani also separated glutamic acid by this method. In all these cases the mould *Penicillium glaucum* was used to effect the separation.

From inactive cystine Neuberger and Mayer separated d-cystine, using *Aspergillus niger* instead of *Penicillium glaucum*, which gave no result with this amino acid.

Not only moulds, but also yeasts can be employed in the separation of optically active compounds as was shown by F. Ehrlich in 1906, who obtained in this way l-alanine, d-leucine, l-valine. Further, amino acids, other than those which occur in nature, can be separated by moulds and yeasts into their components, *e.g.*, n-aminocaproic acid, methylethyl-aminoacetic acid.

It was first shown by E. Fischer, in 1894, that enzymes were specific in their action; thus maltase acts only upon α -glucosides and emulsin only upon β -glucosides. Later, he found that trypsin acted "asymmetrically" upon inactive polypeptides, *e.g.*, alanyl-leucine was hydrolysed in such a way that only the compound composed of d-alanine and l-leucine, the natural isomers, was split up into its constituents, whereas the compound composed of l-alanine and d-leucine was unattacked. Again, inactive leucine ester was found by Warburg to be only partially hydrolysed by trypsin; he obtained l-leucine and d-leucine ester.

Kossel and Dakin, in 1904, found that d-arginine was hydrolysed by the enzyme arginase into d-ornithine and urea, and by using this enzyme Riesser, in 1906, separated dl-arginine, which he had prepared by heating d-arginine with sulphuric acid to 160-180° C. into l-arginine, d-ornithine and urea, the racemic compound being hydrolysed asymmetrically by the enzyme. l-Ornithine can be prepared from the l-arginine by hydrolysis with baryta.

The biological method thus only serves for the preparation of that isomer which does not occur in nature, since the mould or yeast or enzyme destroys the naturally occurring form, leaving the other isomer untouched, or according to Marckwald and Mackenzie, it acts upon the natural isomer more rapidly than upon the other. The method therefore does not lead to the synthesis of the naturally occurring amino acid.

The third method of separating optically active substances by combining them with optically active bases or acids had not been employed with any success until E. Fischer took up this question, the study of the optically active amino acids being his first work upon the chemical constitution of the proteins. The non-success of this method was in all probability due to the small affinity which the simple amino acids themselves have for combining with acids and bases; even the attempts to separate the monoaminodicarboxylic acids, which are fairly strong acids, were not successful.

Hippuric acid, or benzoylglycine, has been known for a long time, and by preparing the benzoyl derivatives of the other amino acids, Fischer found that their acidic character was greatly increased, and that they then combined with the optically active bases brucine, strychnine, cinchonine, morphine, forming stable salts. These salts were much less soluble and their power of crystallising much greater than the salts of the amino acids themselves, and consequently they were more easily isolated; further, they were easily reconverted into the amino acids.

These benzoyl derivatives were prepared by shaking the amino acid with excess of benzoyl chloride in the presence of sodium bicarbonate instead of in the presence of excess of alkali, *i.e.*, by the Schotten-Baumann method, which gave poor and varying yields of the benzoyl compound.

Alanine, aspartic acid, glutamic acid, tyrosine, leucine, phenylalanine and also α -amino-*n*-caproic acid and α -aminobutyric acid have in this way been separated by Fischer into their optically active isomers. To these must be added ornithine which was synthesised by Sørensen in 1903, and separated into *d*- and *l*-ornithine in 1905.

Not only can the benzoyl derivative be employed for this purpose but also the formyl derivative which is prepared by heating the amino acid with anhydrous formic acid at 100° C. These formyl derivatives also give beautifully crystalline salts with the optically active bases, and they possess one great advantage over the benzoyl derivatives, namely, that the formyl group is easily removed by hydrolysis, whereas the benzoyl group requires prolonged heating with a large excess of acid for its removal. The formyl derivative is of enormous advantage also for building up optically active polypeptides, as it admits of the preparation of large quantities of the optically active amino acids.

Fischer and his pupils have thus prepared the optically active forms of leucine, phenylalanine, and valine, and also of phenylaminoacetic acid, and Locquin has prepared, by means of the formyl derivative, *d*-isoleucine.

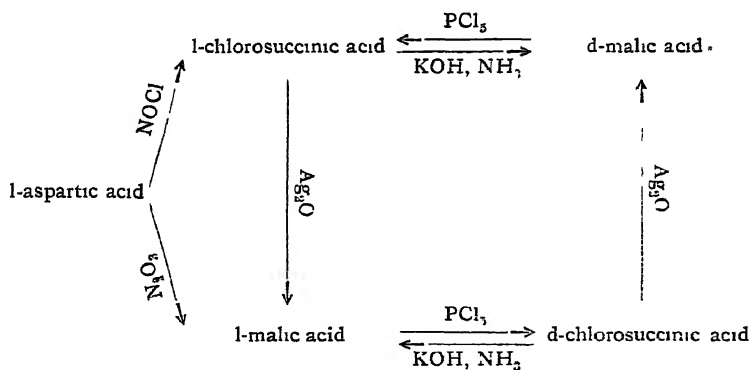
In the case of serine, no separation could be effected by means of

these derivatives, but, by making the p-nitrobenzoyl compound, Fischer and Jacobs obtained d- and l-serine. Isoserine and diaminopropionic acid must also be added to the list of optically active amino acids separated by Fischer and his pupils.

By combining α -aminophenylacetic acid with d-camphorsulphonic acid Betti and Mayer in 1908 separated it into its isomers. This seems to be the first case in which the basic function of an amino acid has been requisitioned for purposes of separation; in all the above cases, the acidic function, by combination with optically active bases, has been made use of.

The separation of serine into its optical antipodes was of the greatest importance, since it has enabled Fischer to correlate together the configuration of d-alanine, l-serine and l-cystine, and also to connect them with d-glucose.

It was first observed by Walden, in 1896, that a change of configuration took place in the conversion of the malic acids into the chlorosuccinic acids and *vice versa*. His results were collected together in 1897 and expressed in the following scheme, in which was included Tilden and Marshall's observations on aspartic acid —

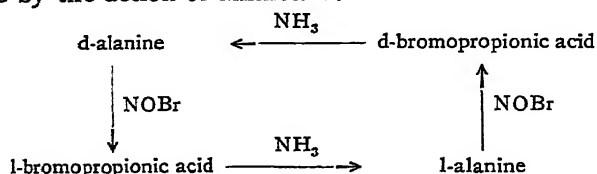


Walden concluded that potassium hydrate and phosphorus pentachloride acted optically normally, *i.e.*, without alteration of the configuration, but that silver oxide, and therefore also nitrous acid and nitrosyl chloride, acted optically abnormally, but as to which of these reactions was really the normal one he was not able to decide. The conclusion was remarkable, since the action of silver oxide takes place in aqueous solutions at a low temperature and the effect of potash in producing racemisation is well known. Still more curious is the supposition that nitrous acid and nitrosyl chloride act optically abnormally.

A similar change in rotation was observed in 1905 by Fischer and

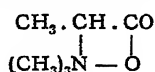
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Warburg in the conversion of alanine into the corresponding halogen fatty acid by nitrosyl bromide and in the reconversion of this compound into alanine by the action of ammonia :—



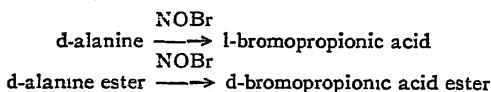
It was termed the "Walden inversion" by Fischer in 1907.

A change in configuration occurs either by the action of ammonia or by the action of nitrosyl bromide. By studying the conversion by the action of ammonia under various conditions, Fischer was able to show conclusively that this reagent behaved optically normally, which result was confirmed by a later experiment upon optically active trimethyl- α -propiobetaine (α -homobetaine),



which he prepared from trimethylamine and d- α -bromopropionic acid, and showed was identical with that prepared by the action of methyl iodide upon d-alanine.

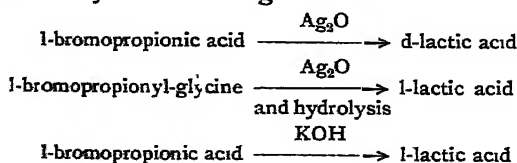
The change produced by nitrosyl bromide was found to be optically abnormal; the following reactions occurred :—



which were confirmed by similar observations upon l-leucine ester, l-phenylalanine ester and on l-aspartic ester. The same reagent can thus sometimes act optically normally and sometimes optically abnormally upon such similar compounds as acid and ester.

Phosphorus pentachloride most probably acts optically normally since it yields products having the same configuration whether it acts upon a hydroxy acid or its ester; further evidence is, however, still required.

Silver oxide behaves like nitrosyl bromide, sometimes normally, sometimes abnormally. The changes



were observed.

It may be concluded that the "Walden inversion" is limited to the reactions between nitrosyl bromide and the amino group and between silver oxide and halogen fatty acid, and is dependent upon the presence of the carboxyl group.

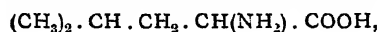
All amino acids would be expected to behave in the same way as leucine, etc., but valine was found by Fischer and Scheibler in 1908 to behave differently:—



that is, the same valine and not its optical antipode was obtained. Either a "Walden inversion" has occurred twice, which is very improbable, or the change into bromovalerianic acid has taken place without a change in the configuration.¹ It appears to be due to the effect of the isopropyl group. In valine,



it is attached directly to the asymmetric carbon atom, in leucine,



a methylene group is present between it and the $\text{CH}(\text{NH}_2)$ group which contains the asymmetric carbon atom

No "Walden inversion" was found to take place when amino acids were converted into the corresponding hydroxy acid by the action of nitrous acid. It was therefore possible to determine the relationship of serine to glyceric acid. d-serine was converted by nitrous acid into a glyceric acid which Neuberg and Silbermann regarded as l-glyceric acid on account of its relationship to l-tartaric acid, but which Neuberg, a little later, stated required confirmation.

In 1907 and 1908 Fischer and Raske correlated together the configurations of l-serine, d-alanine and l-cystine by means of l- α -amino- β -chloropropionic acid,

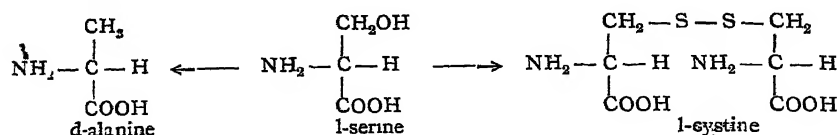


which they obtained from l-serine methyl ester by the action of phosphorus pentachloride and subsequent hydrolysis with hydrochloric acid. By reducing it with sodium amalgam they obtained d-alanine, and by treating it with barium hydrosulphide and oxidising the resulting cysteine, by drawing a current of air through the solution, they obtained cystine.

¹ As this monograph is going through the press a more recent publication by Fischer and Scheibler (Ber., 1908, 41, 2891) points to the probability of the "Walden inversion" occurring twice.

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If l-serine has the configuration represented, d-alanine and l-cystine will have the groups attached to the asymmetric atom arranged in the same order as in l-serine:—



The foundation for the configuration of these amino acids will be obtained if α -amino- β -chloropropionic acid can be converted into aspartic acid, the configuration of which is known from its relationship to malic acid. (d-aspartic acid is converted into d-malic acid by nitrous acid.) The configuration of malic acid can be referred to that of tartaric acid and thence to d-glucose.

Besides the above-mentioned products, several other amino acids have been described as occurring in the protein molecule. Of these, the presence of aminobutyric acid, which would complete the series of monoaminomonocarboxylic acids, was assumed by Schutzenberger, but has not been demonstrated by any of the subsequent investigators. A large number of new products were added to the list by Skraup in 1904, but he has since shown that two of them were mixtures of glycine and alanine. Another amino-oxy acid was described, as also caseanic and caseinic acids, the latter is apparently identical with Fischer and Abderhalden's diaminotrioxydodecanic acid. Another product, diamino-oxysebacic acid, was stated by Wohlgemuth to be a constituent, but its presence as well as that of those described by Skraup has not been definitely proved; they cannot therefore be regarded as units of the protein molecule.

Numerous amino acids—including diamino- and oxyamino-acids—have also been synthesised of recent years by Neuberg and his co-workers and by Sorensen. Our knowledge of these acids should render the task of identifying a new unit in the protein molecule less laborious than it has hitherto been. Their preparation was no doubt due to the possibility of the presence of other units than those above described, which possibility will not be excluded until the quantity of products isolated reaches 100 per cent.

The presence of glucosamine in the protein molecule is also a disputed question; there is no doubt that a carbohydrate containing nitrogen is contained in the glucoproteins in their prosthetic group, but it is doubtful if it is present in the protein part of the molecule, although a

carbohydrate has been obtained from carefully purified proteins containing no prosthetic group, such as crystallised egg-albumin, serum-albumin (Langstein). The fact that the yield of carbohydrate from such a protein becomes smaller the more often it is recrystallised, suggests that the presumably pure protein still contained an impurity; this impurity would be a glucoprotein, which is found in both egg white and serum from which the crystallised proteins are separated, and this would give rise to the carbohydrate.

The constitution of glucosamine, which has been synthesised by Fischer and Leuchs, therefore forms part of the subject of ~~glucoproteins~~.

The constitution of all the amino acids except diaminotrioxydodecanic acid is thus known, and with the exception of histidine they have all been synthesised. The separation of the synthetical compound into its optical antipodes has still to be effected in the case of several of the amino acids.

A brief summary of the discovery of the above amino acids and by whom they were first synthesised is given in Table A. Table B contains the specific rotatory powers of the natural and synthetical substances.

TABLE A.

	Discovered		Racemic "dl" Form Synthesised			Natural Active Form Synthesised	
		in	by	in		by	in
Glycine	Braconnot	1820	Perkin and Duppa	1858	.	..	
Alanine	Schutzenberger	1888	Strecker	1850	d	Fischer	1899
Valine	Weyl		Fittig and Clark	1866	d	"	1906
	v. Gorup-Besanez	1856		1855			
Leucine	Proust	1818	Limpricht	1885	l	"	1900
	Braconnot	1820	Schulze and Likiernik				
Isoleucine	F Ehrlich	1903	Bouveault and Locquin	1905	d	Locquin	1907
Phenylalanine	Schulze and Barbieri	1881	Erlenmeyer and Lipp	1883	l	Fischer and Scholler	1907
Tyrosine	Liebig	1846	Erlenmeyer and Lipp	1883	l	Fischer	1900
Serine	Cramer	1865	Fischer and Leuchs	1902	l	Fischer and Jacobs	1906
Cystine	Wollaston	1810	Erlenmeyer, jun.	1903	l	Fischer and Raske	1908
	Morner	1899				Piutti	1887
Aspartic Acid	Plisson	1827	Dessaignes	1850	l	Fischer	1899
Glutamic Acid	Ritthausen	1866	Wolff	1890	d	Sorensen	1905
Ornithine	Jaffé	1877	Fischer	1900	d		
Arginine	Schulze and Steiger	1886	Schulze and Winterstein	1899	d		
Lysine	Drechsel	1889	Fischer and Weigert	1902	d		
Proline	Fischer	1901	Willstätter	1900	l		
Oxyproline	"	1902	Leuchs (?)		l		
Histidine	Kossel	1896			l		..
Tryptophane	Hopkins and Cole	1901	Ellinger and Flamand	1907	l		
Diaminotrioxo- dodecanic Acid	Skraup Fischer and Ab- derhalden	1904			..		

TABLE B₁.
SPECIFIC ROTATORY POWER OF THE NATURAL AMINO ACIDS

				Observed by
d-Alanine	in water	in alkali	in HCl + 10.3°	Fischer
d-Valine	...		" + 27.9°	Schulze and Winterstein
l-Leucine			" + 16.9°	Schulze
"	in water - 10.8°		in 20% HCl + 15.7°	Ehrlich
d-Isoleucine	" + 9.7°		" + 36.8°	"
l-Phenylalanine	" - 35.3°			Schulze
l-Tyrosine	"		in 21% HCl - 8.5°	Schulze & Bosshard
"	"		in 4% HCl - 15.6°	"
l-Serine	"		" - 12.6°	Fischer
l-Cystine	"		in HCl + 11.6°	"
l-Aspartic Acid		in alkali - 2.4°	in HCl - 22.3 to - 22.4°	Morner
d-Glutamic Acid			in HCl + 25.7°	Fischer
"			" + 30.5°	"
d-Ornithine			" + 31.1°	Schulze & Bosshard
d-Arginine				"
d-Lysine			in HCl + 21.2°	Gulewitsch
l-Histidine			" + 17.5°	Lawrow
l-Proline	in water - 77.4°	in alkali - 83.5°	in HCl - 46.5°	Kossel
l-Oxyproline	" - 81.1°			Fischer
l-Tryptophane	- 33°			"
"	- 30° to - 40°	in $\frac{N}{I}$ alkali + 5.7°	in HCl - 13.5°	Hopkins and Cole
"	in water - 30.3°	in $\frac{N}{2}$ alkali + 6.2°	in HCl + 1.3°	Fischer
l-Diaminotrioxo-dodecanoic Acid	" - 9°			Abderhalden and Fischer and Abderhalden

TABLE B₂.
SPECIFIC ROTATORY POWER OF THE SYNTHETICAL AMINO ACIDS

				Observed by
d-Alanine	in water -	in alkali -	in HCl + 9.7°	Fischer
l-Alanine	"	"	- 10.3°	"
d-Valine	in water + 6.4°		in 20% HCl + 28.7°	"
l-Valine	"		" - 28.7°	"
"	"		" - 27.4°	Ehrlich
d-Leucine	"		" - 15.6°	Fischer and Warburg
l-Leucine	"		" + 15.8°	"
d-Isoleucine	in water + 11.3°		" + 40.6°	Locquyn
l-Isoleucine	" - 10.6°		" - 31.4°	"
d-Phenylalanine	" + 35.1°	...	in 18% HCl + 7.1°	Fischer and Mouneyrat
l-Phenylalanine	"			"
d-Tyrosine	"		in 21% HCl + 8.6°	Fischer
l-Tyrosine	"		" - 8.6°	"
"	"		in 4% HCl - 13.2°	"
d-Serine	in water + 6.9°	...	in HCl - 14.3°	Fischer and Jacobs
l-Serine	" - 6.8°		" + 14.5°	"
d-Cystine	"			"
l-Cystine	"		in HCl - 209.6°	Fischer and Raske
d-Aspartic Acid	"		" - 25.5°	Fischer
l-Aspartic Acid	"	in alkali - 2.3°	"	"
d-Glutamic Acid	"		in HCl + 30.8°	"
l-Glutamic Acid	"		" - 30.1°	"
l-Arginine	"		in HCl - 20.5°	Rieser

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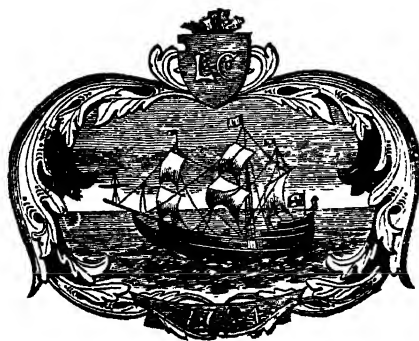
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OF
THE PROTEINS

BY
R. H. ADERS PLIMMER, D.Sc.
ASSISTANT PROFESSOR OF PHYSIOLOGICAL CHEMISTRY IN, AND FELLOW OF
UNIVERSITY COLLEGE, LONDON

IN TWO PARTS
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TO

EMIL FISCHER

THE MASTER OF

ORGANIC CHEMISTRY IN ITS RELATION TO BIOLOGY

GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

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R. H. A. P.
F. G. H.

P R E F A C E.

THE substance Protein, which constitutes the most important part of the material basis of all animal and vegetable life, has naturally attracted the attention and energy of numerous investigators throughout the past century. Progress in the study of this subject, on account of its difficulty, has been exceedingly slow, and it is only of recent years that the discovery of new methods by Emil Fischer has enabled us to increase our knowledge to its present extent. By these methods we have been able to advance from the conception of "albumin" to its actual separation into numerous units, and also to determine their arrangement in the molecule. On this account a monograph embodying the results of the most recent investigations, together with their connections with the work of the other and earlier investigators, needs no excuse for its appearance, as the subject is now being studied in every direction.

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- I { The Chemical Composition of the Protein Molecule.
The Chemical Constitution of its Units.
- II. The Synthesis of the Proteins.

R. H. A. P.

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THE CHEMICAL CONSTITUTION OF THE PROTEINS.

PART II.

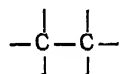
SECTION III.—THE SYNTHESIS OF THE PROTEINS.

Introduction.

It has been recognised since the time of Liebig that the protein molecule is composed of amino acids, but only during the last decades has it been found that these compounds are so numerous and varied in their chemical composition.

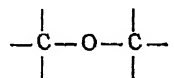
There are various ways in which we can conceive that the amino acids are combined together in the protein molecule. These were summarised and criticised by F. Hofmeister in 1902 as follows:—

I. The carbon atoms can be linked together directly:



Under these conditions the protein molecule would be a huge branched carbon chain, and its degradation into smaller complexes is difficult to explain, and further, such a decomposition by the action of enzymes, *e.g.*, by trypsin, has not yet been observed.

II. The carbon atoms can be linked together by an oxygen atom:



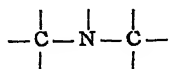
An ether-like combination of the amino acids was suggested by Nasse from the analogy between the hydrolysis of proteins by enzymes and that of the carbohydrates and fats. On account of the small number of hydroxyl groups in the molecules of the amino acids, which is limited to those contained in tyrosine, serine and oxyproline, such a combination can scarcely exist at any rate as the principal method of combination.

An ester-like combination of the carboxyl group of an amino acid with a hydroxyl group for the same reasons is not possible, nor is the acid anhydride method of combination possible. A further reason

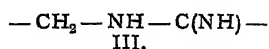
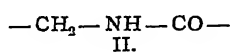
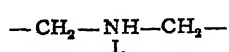
2 THE CHEMICAL CONSTITUTION OF THE PROTEINS

against this mode of combination is the strongly basic character of such compounds, which was first shown by Curtius in the case of glycine ester.

III. The carbon atoms can be linked together by a nitrogen atom :



Several possibilities immediately occur for this mode of combination, of which the three following are the most likely :—



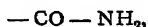
A linking as in Scheme I., which occurs for example in proline, cannot occur to any large extent, since if two amino acids be thus combined together the molecule would become strongly acid in character owing to the free carboxyl groups.

A linking as in Scheme III., which is that of guanidine, occurs in arginine. Only in this compound does such a complex occur in the protein molecule, and therefore such linkings cannot be of the chief importance for the constitution of the molecule.

A large number of important facts support Scheme II. as being the most important for the combination together of the amino acids.

(a) The products of hydrolysis.

A small proportion of the total nitrogen of the protein molecule is liberated on hydrolysis as ammonia; this points to the presence of the acid amide,



form of combination.

The greater portion of the total nitrogen—about 90 per cent.—is present in the products of hydrolysis in the form of amino (NH_2) groups, and the remainder in the form of imino (NH) groups, as in arginine.

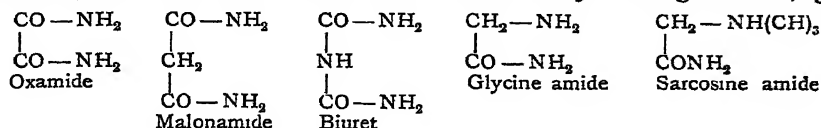
The amino groups are not present in the protein molecule as such, since by the action of nitrous acid on the protein the amount of nitrogen liberated is very small in amount, and in no way corresponds to the amount obtainable if the greater part of the nitrogen be present in the form of amino groups.

It must therefore be assumed that the NH_2 groups of the end products exist in the protein molecule in the form of NH groups.

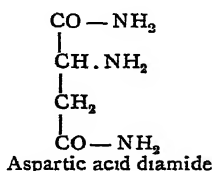
(b) The biuret reaction.

The biuret reaction, which is one of the chief characteristics of a

protein, is, according to Schiff, given by those substances which contain two $\text{CO}-\text{NH}$ complexes, or two $\text{CS}-\text{NH}$ or $\text{C}(\text{NH})-\text{NH}$ complexes, and under certain conditions two $-\text{CH}_2-\text{NH}$ complexes, combined together directly, or by a carbon atom, or by a nitrogen atom, *e.g.*,

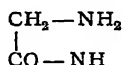


and also

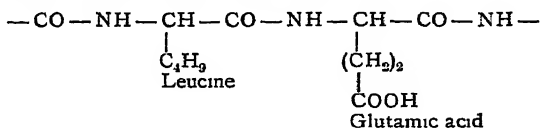


give very intense biuret reactions.

The presence of



groups in the protein molecule is therefore very probable. Such groups occur when amino acids, *e.g.*, leucine and glutamic acid, are combined together in the following way —



and are repeated when another amino acid is again combined in this manner.

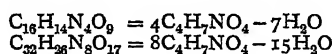
(c) The combination of amino acids by the formation of $\text{CH}_2-\text{NH}-\text{CO}$ groups is also supported by the results obtained in the living body. Hippuric acid $\text{C}_6\text{H}_5.\text{CO}-\text{NH}.\text{CH}_2.\text{COOH}$ is formed from benzoic acid and glycine by the kidney, and the bile acids are also combinations of this nature.

(d) The various results obtained by the condensation together of amino acids, namely, those by Schaal, Grimaux and Curtius, with his biuret base and hippuric acid compounds (see later), many of which give the biuret reaction, support the above supposition, the proof of which has been given by Emil Fischer by his synthesis of the polypeptides where the group $-\text{CO}-\text{NH}-\text{CH}_2-$ occurs repeatedly, and is the chief form of combination in the protein molecule.

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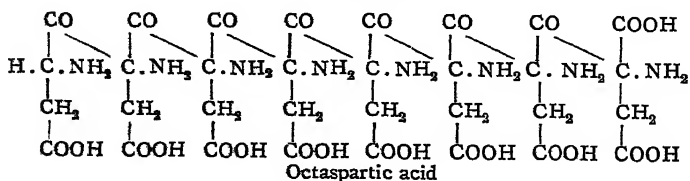
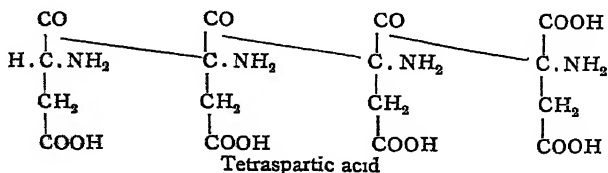
The Condensation Together of Amino Acids.

The earliest investigations upon the condensation together of amino acids were made by Schaal in 1871, who heated asparagine hydrochloride in a current of carbonic acid for three days at 180° C., whereby he obtained a hard white mass, the greater portion of which was insoluble in water and the remainder soluble only with difficulty. The insoluble body was formed by the loss of fifteen molecules of water from eight molecules of aspartic acid, and the other body by the loss of seven molecules of water from four molecules of aspartic acid.—

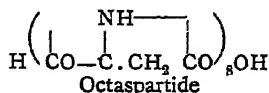
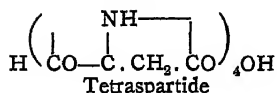


Both compounds were converted into aspartic acid by hydrolysis with baryta water.

J. Guareschi, in 1876, further investigated these substances by determining the amount of silver in the silver salts, but their nature was only demonstrated in 1897-1899 by Schiff. He obtained them by heating aspartic acid, prepared from asparagine and dried at 110° C., for twenty hours at 190-200° C., the yield amounting to 72-75 per cent. Not only were the anhydrides, octaspartide and tetraspartide, as Schiff called these compounds, formed in the process, but also the tetraspartic and octaspartic acids. These acids he also prepared from the anhydrides by hydrolysis with the calculated quantity of cold dilute alkali. From the analysis of their salts, as also their anilides and phenylhydrazides, and from the fact that they gave the biuret reaction which was not observed by Schaal, but pointed out by Grimaux in 1882, he gave these acids the following formulæ:—

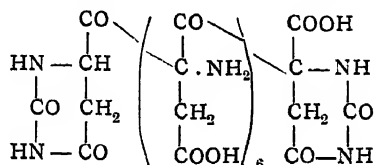


and their anhydrides:—

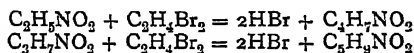


The octaspartic acid was an octobasic acid, its ninth carboxyl group being neutralised by the adjacent NH_2 group.

The researches of Schutzenberger between 1875 and 1880 upon the products of hydrolysis of proteins by the action of baryta water under pressure, led the French chemists to the belief that the proteins were composed of amino acids and urea or oxamide. In 1882 therefore Grimaux heated Schaal's aspartic acid anhydride with urea for two hours at $125\text{--}130^\circ \text{C}$. A thick mass almost entirely soluble in water resulted; its solution was gelatinous and difficult to filter, and it possessed the properties of colloidal substances, behaving very like albumin. This polyaspartic ureide gave the biuret reaction, and was converted by baryta into carbonic acid, ammonia and aspartic acid; it had the formula $\text{C}_{34}\text{H}_{40}\text{N}_{10}\text{O}_{25}$, and consisted of eight molecules of aspartic acid and two molecules of urea. Schiff gave it the formula



In 1888 Schutzenberger, who regarded proteins to be composed of (1) urea and oxamide; (2) leucines, or amino acids of the formula $\text{C}_n\text{H}_{2n+1}\text{NO}_2$, where $n = 6, 5, 4, 3, 2$; (3) leuceines, or amino acids of the formula $\text{C}_n\text{H}_{2n-1}\text{NO}_2$, where $n = 4, 5, 6$, and that there was one molecule of leucine to one molecule of leuceine, prepared the leucines by the action of ethylene dibromide upon the zinc salts of the lower leucines, such as glycine and alanine, according to the equations

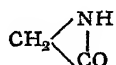


and in 1891 heated a mixture of leucine and leuceine with 10 per cent. urea, carefully dried at 110° , with phosphoric anhydride. He obtained a mass soluble in water, which was precipitated by several volumes of alcohol; it gave the biuret reaction and other protein reactions, and Schutzenberger regarded it as a "pseudo-peptone synthetique".

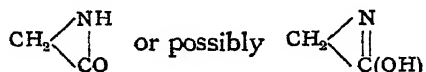
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The Biuret Base.

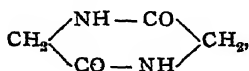
In 1883 Curtius first prepared glycine ester by decomposing glycine ester hydrochloride with silver oxide. It was a colourless, strongly basic oil, very unstable, and only capable of preservation in dry ether. If it were allowed to stand in the air, it underwent decomposition and was converted into an insoluble anhydride,



and a soluble base, which gave the biuret reaction and was called the biuret base. Further investigations upon the nature of these compounds were made by Curtius and Goebel in 1888, who found that the glycine anhydride separated when the ester was allowed to stand for a few days with four volumes of water, and that from the analysis of its silver and copper compounds it had the formula

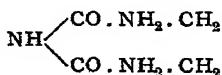


No result could be arrived at concerning the biuret base, which was prepared by keeping pure glycine ester in a sealed tube, when it changed into a white, crystalline mass. Curtius and Schulze, in 1890, by molecular weight determinations found that the formula of the anhydride must be doubled, and it was probably represented by



which was ultimately proved by Fischer and Fourneau in 1901.

This anhydride or biuret base was investigated again in 1894 by Lilienfeld, who prepared it by heating glycine ester with solid potassium bisulphate on the water bath, and who gave it the formula $\text{C}_4\text{H}_9\text{N}_3\text{O}_2$, and the constitution



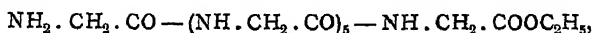
since he also obtained dimethylamine, ethyl ether and carbonic acid in its preparation.

When he heated it with water, Lilienfeld obtained a flocculent precipitate, just as Curtius and Goebel had observed; this formed a gelatinous mass after filtering which contracted like gelatin and behaved, in fact, very like glutin. In a similar manner Lilienfeld condensed

leucine ester and tyrosine ester with glycine ester, whereby he obtained a peptone-like body giving all the principal protein reactions.

Another anhydride of glycine was obtained in 1900 by Balbiano and Trasciatti who heated glycocoll with glycerol; it was a yellowish powder insoluble in all neutral solvents, like the horny substances, and on hydrolysis Balbiano found that it was reconverted into glycine.

Not, however, until 1904 were any further investigations carried out concerning the constitution of the biuret base. Schwarzschild then suggested that it consisted of seven glycine molecules combined together in an open chain, and that it was the ethyl ester of hexaglycyl-glycine of the formula



but Curtius shortly afterwards showed that this was erroneous, and that the body with which Schwarzschild had worked was still a mixture of glycine anhydride and biuret base.

By studying the conditions under which glycine ester was converted into glycine anhydride and biuret base, Curtius showed that, when moisture was excluded as completely as possible, the biuret base with only traces of glycine anhydride was formed, and that the amount of glycine anhydride produced increased with the amount of water present. Thus, if pure glycine ester were kept in the absence of air, it solidified in a few days and the mass contained biuret base with 23-24 per cent. of glycine anhydride; if glycine ester were boiled with dry chloroform, 12 per cent. of glycine anhydride was formed, but if perfectly pure glycine ester were mixed with about a third of its volume of absolute ether and left for some weeks it was almost completely converted into biuret base, only 1 per cent. of glycine anhydride being present. The analysis, molecular weight, properties and reactions of the biuret base showed conclusively that it was triglycyl-glycine ester, *i.e.*, a tetraglycyl compound of the formula

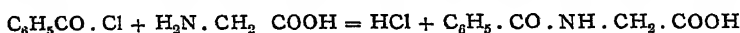


It was observed by Curtius and Gumlich that the biuret base when heated *in vacuo* to 100° C. lost alcohol, and that it was converted into an anhydride, most probably octoglycine anhydride, so that from glycine ester quite complex substances can be obtained. Such substances have also been obtained by Emil Fischer and his pupils by the condensation of amino acid and polypeptide esters (see below).

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The Linking Together of Amino Acids.

By the action of benzoyl chloride upon the silver salt of glycine, Curtius, in 1881, obtained in addition to the expected hippuric acid



two other acids of higher molecular weight. One of these was hippuryl-glycine or benzoyl-glycyl-glycine,



as was proved by the study of its salts, its ethyl ester and amide, and by its hydrolysis into hippuric acid and glycine. It was the first definite compound known which contained two amino acid residues combined together.

The constitution of the other acid, called the γ -acid, could not be determined; it had the formula $\text{C}_{10}\text{H}_{12}\text{N}_3\text{O}_4$, was soluble with difficulty in water and gave the biuret reaction. It was formed in larger quantities when hippuric ester was fused with glycine, but under these conditions another compound—benzoyl-bisglycyl-glycine—was formed at the same time, so that in this way a series of compounds resulted, each succeeding member containing a glycyl- $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CO}$ - group more than the preceding one.

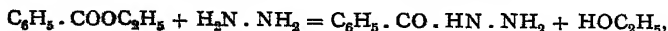
Further investigations upon the constitution of these compounds were only carried out in 1904 by Curtius and Benrath, who found that the γ -acid from the analysis of its silver salt, ester, etc., had the formula $\text{C}_{19}\text{H}_{24}\text{N}_6\text{O}_8$, and that it was benzoyl-pentaglycyl-glycine,



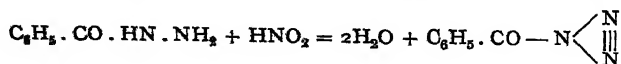
its ester being identical with the compound synthesised by Curtius and Wustenfeld (see below).

Two other compounds—hippuryl-glycine and benzoyl-triglycyl-glycine—were found to be formed by fusing together hippuric ester and glycine, but not the previously isolated benzoyl-diglycyl-glycine. Longer chains than the six membered γ -acid are not believed by Curtius and Benrath to be formed in this reaction.

In 1890 Curtius, by the action of hydrazine upon benzoyl chloride, benzamide or benzoic ester, obtained benzoylhydrazine,



which, when treated with nitrous acid, gave benzoylazoimide,



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This compound was also prepared from benzoyl-triglycyl-glycine azide and glycine ester.

The further lengthening of the chain by means of the azide of benzoyl-tetraglycyl-glycine could not be accomplished since this compound could not be prepared, but the next member of the series, benzoyl-pentaglycyl-glycine ester,



was prepared from benzoyl-triglycyl-glycine azide and glycyl-glycine ester. This was identical with the original γ -acid of 1883, of which Curtius and Benrath had determined the constitution.

By condensing the biuret base, which in the meanwhile had been proved to be triglycyl-glycine ester with hippurazide, Curtius and Levy obtained again the former benzoyl-tetraglycyl-glycine, and by condensing it with hippuryl-glycine azide they obtained benzoyl-pentaglycyl-glycine ester, and thus by a less circuitous method attained to the same compound as they had prepared from hippurazide. Further lengthening of the glycyl chain has not as yet been carried out by this method, but the method has been adapted by Curtius and Lambotte to the formation of alanine chains, namely:—

Hippuryl-alanine, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$ from hippurazide and α -alanine

II $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$ from hippuryl-alanine azide and α -alanine.

Hippuryl-alanyl-alanyl-alanine, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$ from hippuryl-alanyl-alanine azide and α -alanine.

These all contain the glycine residue as well as the alanine residue in their molecule; in order to eliminate the glycine residue and obtain compounds without the glycine radical, Curtius and van der Linden prepared the following compound:—

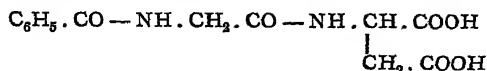
Benzoyl-alanyl-alanine, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$ from benzoyl-alanine azide, which was prepared from benzoyl-alanine, as obtained by Fischer's method, and alanine.

Benzoyl-alanine azide was also combined with glycine, and this radical introduced at the end of the chain; thus they prepared

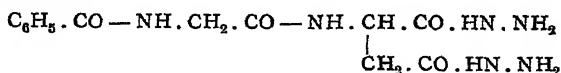
Benzoyl-alanyl-glycine, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$ from benzoyl-alanine azide and glycine.

Benzoyl-alanyl-glycyl-glycine, $\text{C}_6\text{H}_5 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$ from benzoyl-alanyl-glycine azide and glycine, or benzoyl-alanine azide and glycyl-glycine.

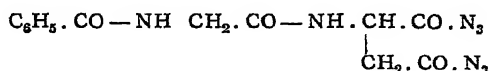
The behaviour of hippurazide with the dibasic aspartic acid was investigated by Th. and H. Curtius in order to build up chains containing this amino acid. By the action of hippurazide upon aspartic acid in alkaline solution they obtained hippuryl-aspartic acid:—



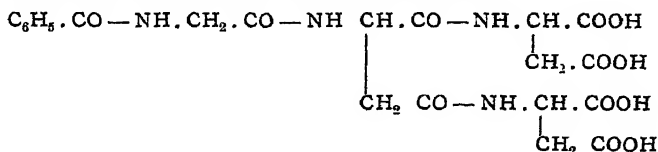
The ester of this compound was converted into the hydrazide by means of hydrazine,



from which hippuryl-aspartic acid azide was obtained by the action of nitrous acid:—

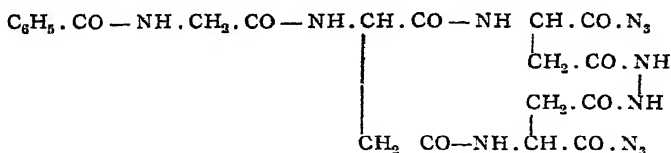


This reacted in ethereal solution with aspartic ester yielding hippuryl-asparagyl-aspartic ester from which the free acid,

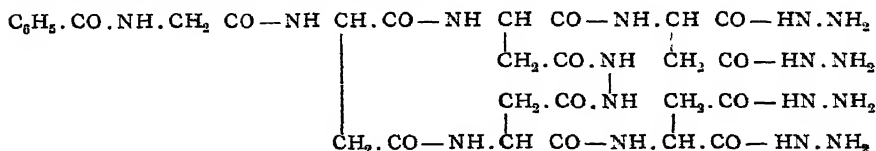


was obtained by saponification with baryta.

The hydrazide of this compound was then prepared from the ester in the usual manner, and from this the azide, which did not, however, possess the normal structure, but that of the hydrazi-azide,

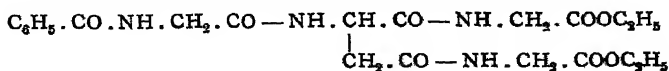


The condensation product of this compound with aspartic ester was not isolated, but the complex hippuryl-disaspartyl-aspartic acid hydrazi-hydrazide, its hydrazine derivative,



was obtained when the condensation product was treated in alcoholic solution with hydrazine hydrate.

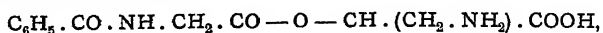
Just in the same way hippuryl-aspartyl-glycine ester,



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resulted when hippuryl-aspartic acid azide was combined with glycine ester.

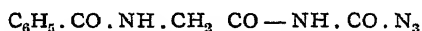
In conjunction with Gumlich, Curtius has investigated the linking of hippurazide with β -amino- α -oxypropionic acid and with β -aminobutyric acid. With the former compound, the combination took place with the hydroxyl group instead of with the amino group, hippuryl- α -oxy- β -aminopropionic acid,



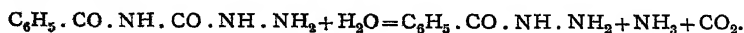
being formed. With the latter, by the usual series of reactions they prepared hippuryl- β -aminobutyric acid and hippuryl- β -aminobutyryl- β -aminobutyric acid.

Curtius and Muller have also prepared hippuryl- γ -aminobutyric acid and hippuryl- β -phenyl- α -alanine, compounds of no great interest since these amino acids do not occur in the protein molecule. They show, however, that not only can α -amino acids be combined together by the azide method, but also β - and γ -substituted amino acids.

In order to build up chains containing the carbamic acid radical, $\text{NH} \cdot \text{CO}$, just as Curtius and his co-workers have built up chains containing glycyI, $\text{NH} \cdot \text{CH}_2 \cdot \text{CO}$, alanyl and asparagyl radicals, Curtius and Lenhard, in 1904, proposed to make use of the azide of hippuryl-carbamic acid,



This compound, however, was unavailable, since sufficient quantities of hippuryl urea, which Curtius had formerly prepared from hippuric ester and urea, could not be obtained from hippurazide and urea. They therefore attempted to make the azide of benzoylcarbamic acid by the action of hydrazine on benzoylurea, but the only product which they obtained was the hydrazide of benzoic acid. The benzoic acid radical is therefore very easily eliminated from the urea molecule, the molecule of benzoylcarbamic acid hydrazide, being hydrolysed according to the equation



This non-success led them to attempt to combine phenylcarbamic acid azide $\text{C}_6\text{H}_5 \cdot \text{NH} \cdot \text{CO} - \text{N}_3$, which Curtius and Hofmann and Curtius and Burkhardt had described in 1896 and 1898, with urea, but again the desired result was not achieved, nor could a combination of this compound with biuret be effected.

It followed therefore that acid radicals cannot be combined with urea by the acid azide reaction.

If, however, glycine were used instead of urea for combination with the azide of phenylcarbamic acid phenylcarbamino glycine resulted, which was identical with the compound prepared by Paal in 1894 from phenylisocyanate and glycine.

With this compound Curtius and Lenhard continued the lengthening of the chain by the azide reaction, and obtained

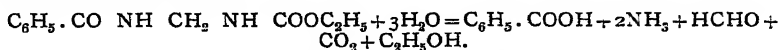
Phenylcarbamino glycyl-glycine, $C_6H_5 \cdot NH \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_2 \cdot COOH$, and phenylcarbamino diglycyl-glycine, $C_6H_5 \cdot NH \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_2 \cdot CO-NH \cdot CH_2 \cdot COOH$.

These compounds and their various derivatives prepared by Curtius and his pupils are white crystalline compounds, for the most part soluble with difficulty in cold water. Some of them give the biuret reaction, but others do not, in particular the less complex compounds where the influence of the acid radical inhibits the reaction, although the compounds possess the exact conditions, as determined by Schiff, for the positive exhibition of the reaction

The reactions given by the azides with alcohol, ammonia, aniline, etc., are of greater interest and may therefore be briefly summarised.

By the action of ammonia the acid azides are either completely saponified into the corresponding acids, or by a rearrangement in the molecule, they are converted into derivatives of urea. With the dibasic acids both possibilities may occur at the same time, and the resulting compound is half acid amide and half urethane. Subsequent hydrolysis clearly shows the nature of the component amino acid chain. Thus, hippuryl urethane is formed from hippurazide and alcohol,

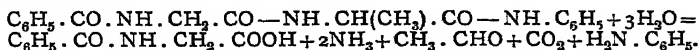
$C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CO \cdot N_3 + C_2H_5OH = C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot NH \cdot COOC_2H_5 + N_2$,
and on hydrolysis it is converted into benzoic acid, ammonia, carbonic acid and formaldehyde:—



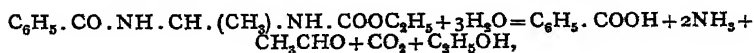
The reaction therefore leads to the formation of formaldehyde from glycine. Hippuryl alanineazide and aniline give the following urea derivative:—



which, on hydrolysis, breaks down into hippuric acid, ammonia, acetaldehyde, carbonic acid and aniline:—

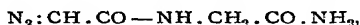


The same products are obtained when the urethane derivative, obtained from benzoylalanine azide and alcohol, is hydrolysed:



The further work, published in 1906, of Curtius and his pupils is concerned with the action of nitrous acid upon the polyglycine compounds. diazoacetyl glycine ester $N_2 : CH . CO - NH . CH_2 . COOC_2H_5$ was formed by the action of nitrous acid upon glycyl-glycine ester hydrochloride and diazoacetyl glycyl-glycine ester $N_2 : CH . CO - NH . CH_2 . CO - NH . CH_2 . COOC_2H_5$ by its action upon diglycyl-glycine ester hydrochloride.

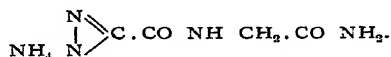
By the action of ammonia on the first body diazoacetyl glycine amide,



was formed, and on the latter diazoacetyl glycyl-glycine amide,

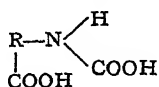


These are yellow substances, but when excess of ammonium hydroxide or liquid ammonia is added to the esters colourless substances are obtained, they were at first regarded as azomethane derivatives, but have since been shown to be the ammonium salts, *e.g.*, of isodiazoacetyl glycine amide,

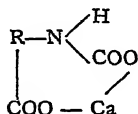


Combinations of Amino Acids with Carbonic Acid.

The sodium and barium salts of the monoamino acids have a strongly alkaline reaction and are highly dissociated salts. If carbonic acid be passed into the solution of the barium salt, barium carbonate is not, as would be expected, immediately formed; the solution remains clear, and only after a short time, when the solution becomes saturated with carbonic acid, does it become cloudy and barium carbonate gradually separates out; the separation of barium carbonate is hastened by heating. This phenomenon is due, as was shown by Siegfried in 1905, to the formation of salts of carbamino acids of the general formula



i.e., to the formation of a dibasic acid of which the calcium salt,



is soluble with difficulty in ice-cold water and alcohol. Similar compounds are formed with the dibasic aspartic and glutamic acids and with the diamino acids. In aqueous solutions also the free carbamino acid is formed. The reaction may serve, as Siegfried pointed out in 1906, for the separation of amino acids from their solutions.

Siegfried and Neumann, in 1908, showed that there was a distinct regularity in the fixation of carbonic acid by amino acids; the amino groups of the aliphatic amino acids were quantitatively converted into carbamino groups; in histidine and arginine only the amino group of the side chain, not the nitrogen atoms of the rings, reacted with carbonic acid to form carbamino groups.

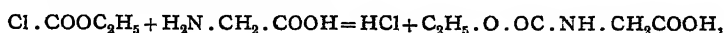
Glycyl-glycine was also found by Siegfried to react with carbonic acid in the presence of barium hydrate with the formation of the barium salt of glycyl-glycine carbamino acid, which on heating was converted into barium carbonate and glycyl-glycine. Further, Siegfried and Liebermann have shown that the peptide linking in the polypeptides reacts to a certain extent, and by this means they hope to obtain an idea of the constitution of the various peptones which Siegfried has isolated from proteins by the action of trypsin.

Not only do the amino acids react with carbonic acid in the presence of calcium salts, but also peptones and the proteins of serum; this may

explain certain of the phenomena concerning the presence of carbonic acid in blood and in working muscle; a protein carbonic acid compound may be formed which can give rise to carbonic acid without taking up oxygen.

Siegfried's results with glycine and glycyl-glycine have been confirmed by Leuchs, who, in addition, has investigated the combinations of amino acids and of polypeptides with carbonic acid which were prepared by Fischer and his pupils by combination with chlorocarbonic ester (see later).

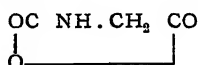
Carbethoxylglycine which was obtained by combining together chlorocarbonic ester with glycine,



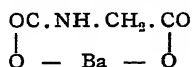
even by careful hydrolysis could not be converted into the free acid, decomposition always occurring with the formation of glycine and carbonic acid; Leuchs, however, in 1907, obtained the free acid indirectly in the following manner: Carbethoxylglycine was converted into its acid chloride,



by the action of thionylchloride, and this compound, when heated, lost ethyl chloride and was changed into the anhydride,



which, when warmed with water to 15° C., decomposed into glycine and carbonic acid, but, when treated with the calculated quantity of baryta, yielded the barium salt of glycine carboxylic acid,



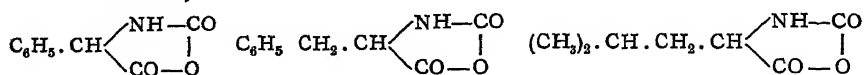
This was identical with the barium salt obtained by Siegfried from glycine, carbonic acid and barium hydrate.

It is of interest to observe that Leuchs found that the anhydride, when treated with a small quantity of water, gave an anhydride of glycine which was not identical with diketopiperazine, but possibly the same substance which Balbiano and Trasciatti (p. 7) obtained by heating glycine with glycerol, or as that obtained by Curtius from the biuret base (p. 7).

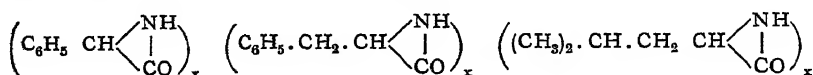
Leuchs and Geiger, in 1908, obtained the anhydrides of C-phenyl-aminoacetic acid, of phenylalanine and of leucine in the same way by heating the acid chlorides of the carbomethoxyl derivatives, which were

18 THE CHEMICAL CONSTITUTION OF THE PROTEINS

prepared by the action of thionyl chloride, whereby methyl chloride was eliminated,

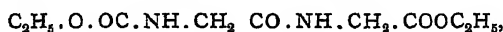


and then warming in the presence of traces of water, when carbon dioxide was evolved with the formation of the anhydrides,

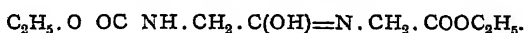


Carbethoxyl-glycyl-glycine ester was found by Fischer to yield on hydrolysis the free glycyl-glycine carboxylic acid, from which, on esterification, an ester was obtained, which was isomeric with the original carbethoxyl-glycyl-glycine ester. This acid was extremely stable in comparison with the glycyl-glycine-N-carboxylic acid obtained by Siegfried in 1906 and also by Leuchs.

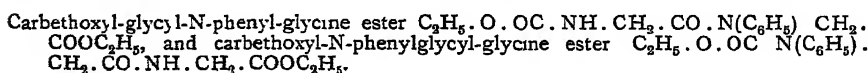
The difference between these compounds was shown by Leuchs and Manasse, in 1907, to be due to the fact that the original ester, which has the lactam formula and belongs to the α -series,



undergoes a transformation during hydrolysis and is converted into the acid having the lactim formula and belonging to the β -series,

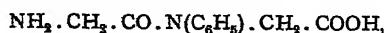


This was proved by the study of the phenyl derivatives,

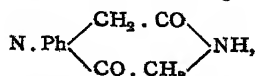


The former was prepared from carbethoxyl-glycyl chloride and phenylglycine ester, the latter from carbethoxyl-N-phenylglycyl chloride and glycine ester.

Owing to the substitution of the hydrogen atom by phenyl in the position represented in carbethoxyl-glycyl-N-phenyl-glycine ester, no transformation into the lactim form can take place. On hydrolysis it yielded the dipeptide glycyl-phenylglycine, with loss of carbonic acid,

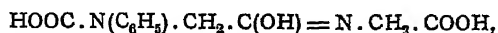


and this dipeptide was converted on heating into the diketopiperazine,

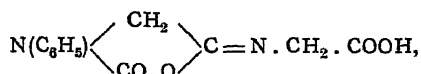


which was also obtained from chloracetylphenylglycine and ammonia.

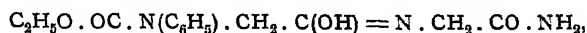
Carbethoxyl-N-phenyl-glycyl-glycine ester on hydrolysis yielded the acid



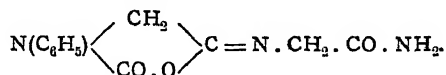
which did not lose carbon dioxide, and was analogous to Fischer's glycyl-glycine carboxylic acid. Phenyl-glycyl-glycine carboxylic acid easily forms the lactone



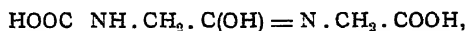
as do also its derivatives; thus carbethoxyl-N-phenyl-glycyl-glycine ester when treated with ammonia yields the amide



which loses alcohol at 220° and forms the lactone



The stability of glycyl-glycine carboxylic acid obtained from carbethoxyl-glycyl-glycine ester is therefore due to its having the lactim formula,



whereas the instability of glycyl-glycine carboxylic acid obtained from glycyl-glycine and carbonic acid is due to the lactam formula,



Further proof for these formulæ is given by Siegfried's experiments in which he showed that the peptide linking in polypeptides, which have the lactam formula, could also combine with carbonic acid, whereas glycyl-glycine carboxylic acid which has the lactim formula did not combine with carbonic acid.

Combinations of Amino Acids with Ammonia.

The amides of amino acids, such as glycynamide,

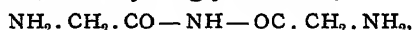


the compounds prepared by Curtius, and the polypeptides (later) of Emil Fischer, such as glycyl-glycine,

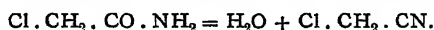


are combinations of amino acids in which one carboxyl group only is attached to the ammonia residue.

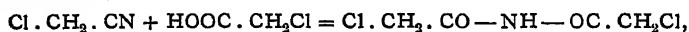
A compound, in which two carboxyl groups of amino acids are attached to ammonia, namely, diglycinimide,



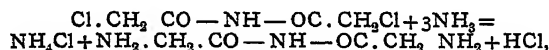
was prepared in 1907 by Bergell. Starting from chloracetamide he obtained chloracetonitrile by heating it with phosphoric anhydride:—



From this compound dichlorodiacetamide resulted in its interaction with chloracetic acid,

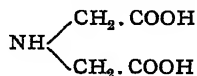


and on treating it with ammonia, diglycinimide hydrochloride was obtained,

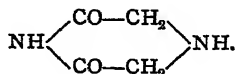


from which the free base was prepared by means of silver oxide as a crystalline substance of a basic character

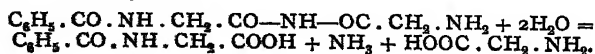
Bergell continued his work on this substance in conjunction with Feigl. Diglycinimide was stable to acids and to the weak alkalies, magnesia and sodium bicarbonate, but it was converted by caustic alkalies and by baryta into ammonia and an acid of the constitution



which was identical with "diglykolamidsäure" prepared in 1862 by Heintz. Its formation probably took place through the intermediate ring compound,



It did not give rise to ammonia and glycine as was expected; its benzoyl derivative, however, on hydrolysis was converted into hippuric acid, ammonia and glycine:—



The Polypeptides.

Our knowledge of the structure of the protein molecule has been given us by the systematic researches of Emil Fischer and his pupils which were commenced in 1901, the combinations together of the amino acids being termed the polypeptides. This designation is in imitation of that of the carbohydrates, where we differentiate between mono-, di-, tri-, poly-saccharides, and it retains the word peptone, on account of the very similar properties of these substances to peptone, which most probably consists of a mixture of polypeptides.

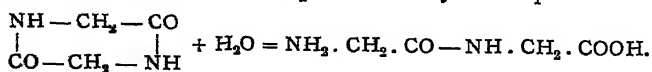
Three general methods have been devised for the synthesis of the polypeptides which are best described separately.

Method I. Synthesis from the Esters.

The ester of glycine was first prepared, as previously mentioned, by Curtius in 1883, and it was observed by Curtius and Goebel, in 1888, that the ester lost alcohol and was converted into 2, 5-diketo- or diacipiperazine.

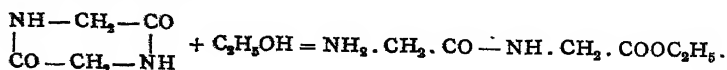
Similar compounds—leucinimide and lactimide—had been obtained from leucine and alanine. These anhydrides form the starting-point in the synthesis of the polypeptides by this method, and they are best obtained by heating the ester of the amino acids in a sealed tube to 150-180° C. for some hours.

Fischer and Fournau, in 1901, found that 2, 5-diketopiperazine, or glycine anhydride, as it is now best termed, was converted by boiling with concentrated hydrochloric acid into the hydrochloride of an amino acid of the formula $C_4H_8N_2O_3$, from which they obtained the free acid, by treatment with the calculated quantity of caustic soda, or by means of silver oxide. Its formation is represented by the equation



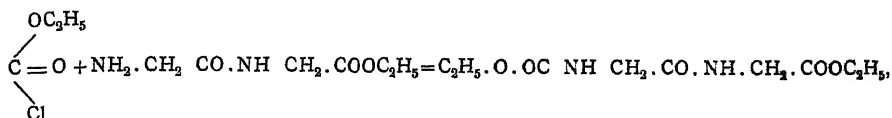
The compound is the first anhydride of glycine, and was termed glycyl-glycine, the group $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CO}$ being called the glycyl group.

By treating glycine anhydride with alcoholic hydrochloric acid, glycyl-glycine ester resulted :—

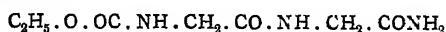


Both the free acid and its ester have a great tendency to become reconverted into glycine anhydride, and both compounds are characterised by

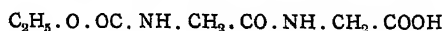
the great reactivity of the NH_2 group; thus, with phenylisocyanate they both yield the compound $\text{C}_6\text{H}_5 \cdot \text{NH} \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$, and the ester gives with ethyl chlorocarbonate, carbethoxyl-glycyl-glycine ester:—



from which the amide

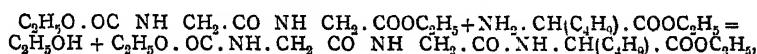


is obtained by the action of ammonia, and the free acid



by careful hydrolysis with soda.

Carbethoxyl-glycyl-glycine ester, when heated with leucine ester, yielded carbethoxyl-glycyl-glycyl-leucine ester,



a compound which contains three amino acids combined together and was the first known representative of a tripeptide.

Carbethoxyl-glycyl-glycine amide and carbethoxyl-glycyl-glycyl-leucine ester give the biuret reaction as would be expected from the researches of Schiff in 1900, who found that glycine amide $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH}_2$ also gave the reaction.

In the same way alanyl-alanine and alanyl-alanine ester, which yielded carbethoxyl-alanyl-alanine ester when treated with ethyl chlorocarbonate, can be obtained from alanine anhydride, and leucyl-leucine from leucine anhydride or leucinimide, which was first obtained in 1849 by Böpp, and regarded as occurring in the protein molecule, by hydrolysis with hydrobromic acid.

The condensation together of other amino acids in this way by heating their esters is accompanied by difficulties. The diketopiperazine ring is not easily split open by means of acid, and although Fischer, in 1905, discovered that the diketopiperazine could be converted into the dipeptide somewhat easily by treatment with the equimolecular quantity of caustic soda in 10-15 minutes at the ordinary temperature, whereby glycyl-glycine and alanyl-alanine could be easily prepared, yet, in other cases, such as that of leucine anhydride, the anhydride was very resistant to alkali. It appears that the stability of the diketopiperazine ring is connected with the nature of the alkyl groups attached to it, and that there is here another instance of steric hindrance.

The dipeptides of the oxy- and diamino acids have so far only been prepared by this method by Fischer and Suzuki; here, the methyl esters of the amino acids were found to be most easily converted into the anhydrides, and hydrolysis by alkali proceeded readily. The compounds thus obtained can be seen from the accompanying list.

Several of the dipeptides are most readily prepared in this way, and they have been employed in the synthesis of more complex polypeptides. The method, however, does not lend itself to the preparation of higher polypeptides, but it will be observed that pentaglycyl-glycine and another compound, probably octaglycine anhydride, have been prepared by heating the methyl ester of diglycyl-glycine. The various compounds isolated by Curtius and his pupils, such as glycine anhydride and the biuret base, have been obtained from glycine ester.

It must be noted that anhydrides are also formed when the dipeptides, prepared by the other methods, are heated to their melting-points, *e.g.*, leucyl-proline anhydride from leucyl-proline.

Mixed anhydrides, as for example glycyl-alanine anhydride, cannot be obtained by heating a mixture of the esters, where a complex mixture would result, but they are easily prepared by the action of ammonia upon the esters of the dipeptides. These compounds, of which several have now been prepared, are of great importance as they serve for the isolation of dipeptides from a mixture of polypeptides and amino acids (see polypeptides isolated from proteins). On hydrolysis they yield a mixture of the two dipeptides, composed of the amino acids of which they are built up. Thus glycyl-l-tyrosine anhydride yielded glycyl-l-tyrosine and l-tyrosyl-glycine. The latter compound is the first example of a polypeptide containing the tyrosine radical as the acyl group; in all the other polypeptides in which tyrosine is present it stands at the end of the chain.

POLYPEPTIDES SYNTHESISED BY METHOD I.

Simple Polypeptides.

Glycine ester	→ glycine anhydride	→ glycyl-glycine.
Alanine ester	→ alanine anhydride	→ alanyl-alanine.
Leucine ester	→ leucine anhydride	→ leucyl-leucine.
Diaminopropionic acid ester	→ diaminopropionic acid anhydride.	
Histidine ester	→ histidine anhydride	→ histidyl-histidine
Lysine ester	→ lysine anhydride	→ lysyl-lysine.
Arginine ester		→ arginyl-arginine (?)
Serine ester	→ serine anhydride	→ seryl-serine.
Iso-serine ester		→ isoseryl-isoserine.
Tyrosine methyl ester	→ tyrosine anhydride	→ tyrosyl-tyrosine.
Aspartic acid methyl ester	→ aspartic acid anhydride	→ diketopiperazine diacetic diamide
	2-5-diketopiperazine, 3-6- diacetic acid	by action of ammonia.
Diglycyl-glycine methyl ester	→ pentaglycyl-glycine ester	→ pentaglycyl-glycine.
ester	→ octaglycine anhydride (?)	
l-alanyl-glycyl-glycine methyl ester	→ l-alanyl-diglycyl-l-alanyl-glycyl-glycine.	
	Leucyl-alanine anhydride	} from dipeptide
	Leucyl-proline anhydride	

Mixed Polypeptides.

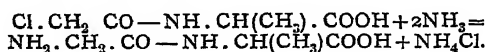
Chloracetyl alanine ester	→ Glycyl-alanine anhydride	→ glycyl-alanine. → alanyl-glycine.
Chloracetyl tyrosine ester	→ glycyl-l-tyrosine anhydride	→ glycyl-l-tyrosine. → l-tyrosyl-glycine.
Glycyl-l-phenylalanine ester	→ glycyl-l-phenylalanine anhydride	→
l-phenylalanyl-glycine ester	→	→
Leucyl-glycine ester	→ leucyl-glycine anhydride	→ leucyl-glycine. → glycyl-leucine.
Glycyl-aspartic acid ester	→ glycyl-aspartic acid anhydride	
	leucyl-alanine anhydride	→ leucyl-alanine. → alanyl-leucine.
Phenylalanyl-glycine ester	→ Phenylalanyl-glycine anhydride.	
Valyl-glycine ester	→ Valyl-glycine anhydride.	
Valyl-alanine ester	→ Valyl-alanine anhydride.	

II. Synthesis of Polypeptides by Means of the Halogen Acyl Compounds.

E. Fischer and E. Otto first described this method of synthesising polypeptides in 1903. Just as an ordinary acyl radical can be combined with an amino acid, *e.g.*, in the preparation of benzoylalanine, so also can a halogen substituted acyl radical be combined with an amino acid. The subsequent action of ammonia upon this compound replaces the halogen atom by the amino group and a dipeptide results, thus:—

Chloroacetylchloride and alanine yield chloroacetylalanine,

$\text{Cl} \cdot \text{CH}_2 \cdot \text{COCl} + \text{NH}_2 \cdot \text{CH}(\text{CH}_3)\text{COOH} = \text{Cl} \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3)\text{COOH} + \text{HCl}$,
from which, by the action of ammonia, glycyl-alanine is obtained:—



In practice this reaction can be carried out in two ways:—

1. By the action of the halogen acylchloride upon the alkaline solution of the amino acid. This reaction proceeds well with the higher acylchlorides which are not rapidly acted upon by water, but with the lower acylchlorides it must be carried out at a very low temperature, and the yields even then are in many cases very poor.

2. By the action of the halogen acylchloride upon the ester of the amino acid in anhydrous solvents, such as ether, chloroform, petroleum ether. In this reaction two molecules of amino acid ester are required for one molecule of halogen acylchloride, since half the ester is removed from the reaction as ester hydrochloride. In order to prevent this, the reaction may be carried out in the presence of alkali or alkali carbonate. Subsequent saponification of the ester follows this operation, and loss results by the action of alkali on the halogen acyl radical. This method is only used when the reaction gives bad yields in aqueous solution.

Several halogen acylchlorides are necessary for introducing the various amino acid radicals. These are:—

Chloroacetyl-chloride for the introduction of the glycyl radical.

α -Bromopropionyl-chloride for the introduction of the alanyl radical.

1- α -Bromopropionyl-chloride for the introduction of the d-alanyl radical.

α -Bromobutyryl-chloride for the introduction of the α -aminobutyryl radical.

α -Bromisocaprolyl-chloride for the introduction of the leucyl radical

α -Bromophenylacetyl-chloride for the introduction of the phenylglycyl radical.

α -Bromo-hydrocinnamyl-chloride for the introduction of the phenylalanyl radical.

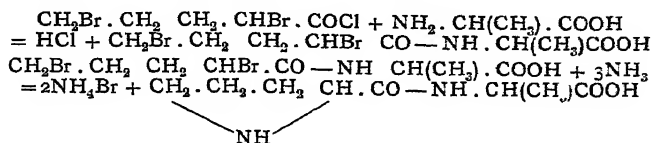
Phenyl-bromopropionyl-chloride for the introduction of the phenylalanyl radical.

α - β -Dibromovaleryl-chloride for the introduction of the prolyl radical.

Fumaryl-chloride for the introduction of the asparagyl radical.

The introduction of the prolyl group into an amino acid by means

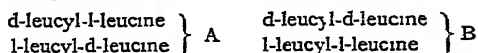
of α - δ -dibromovaleric acid chloride reminds us of the synthesis of proline, where when the compound is treated with ammonia in order to exchange the Br atoms for NH_2 , ammonia is lost and ring formation occurs. Prolyl-alanine is prepared as follows :—



In order to introduce the asparagyl group into an amino acid, chlorosuccinyl chloride, the corresponding halogen-acyl chloride, cannot be employed, since on treatment with ammonia it yields fumaryl derivatives. These, however, when heated with strong ammonia again take up ammonia forming the asparagyl compound, and hence can be employed for this purpose.

These radicals can be introduced into all the simple mono-amino acids, such as alanine, leucine, tyrosine, etc.; also into cystine and the dicarboxylic acids when the compounds such as dialanyl-cystine and asparagyl-dialanine are formed. They can also be introduced into the molecule of a di, tripeptide, etc., as can be seen from the appended list of polypeptides synthesised by this method, which, however, only allows of the chain of amino acids being lengthened on one side, namely, at the amino group end.

The majority of the polypeptides synthesised by this method are optically inactive, but the optically active compounds can also be prepared by employing the optically active halogen-acyl chloride. As previously described under the optically active amino acids, these compounds undergo the Walden inversion, the method therefore allows of the whole of an inactive amino acid being employed for the synthesis of an optically active polypeptide, thus dl-leucine after separation into d-leucine and l-leucine can be converted into l-leucyl-l-leucine by preparing the d-bromisocaprolyl chloride from the d-leucine and combining it with l-leucine; treatment with ammonia gives l-leucyl-l-leucine as the compound undergoes the Walden inversion. The four isomers



can be thus prepared. The A compound is the former inactive leucyl-leucine. Also l-phenylalanyl-glycine was obtained from d-phenyl- α -bromopropionylchloride and glycine, and glycyl-l-phenylalanine from chloracetylchloride and l-phenylalanine, the dl-phenylalanine used having been separated into its optical isomers by means of its formyl compound.

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POLYPEPTIDES SYNTHESISED BY METHOD II.

Dipeptides.

Inactive.	Optically Active
Glycyl-alanine.	Glycyl-l-tyrosine.
Glycyl-phenylalanine.	Glycyl-d-alanine
Glycyl-leucine.	Glycyl-d-tryptophane.
Glycyl-asparagine.	Glycyl-l-phenylalanine.
	Glycyl-3, 5-diiodo-l-tyrosine.
Alanyl-glycine.	dl-Alanyl-d-alanine.
Alanyl-alanine.	l-Alanyl-glycine.
Alanyl-leucine A.	d-Alanyl-d-alanine
Alanyl-leucine B.	d-Alanyl-l-leucine.
Alanyl-phenylalanine.	d-Alanyl-d-tryptophane.
α -Aminobutyryl-glycine.	
α -Aminobutyryl-aminobutyric acid A.	
α -Aminobutyryl-aminobutyric acid B.	
Valyl-glycine.	
Valyl-alanine.	
Leucyl-glycine.	l-Leucyl-l-tyrosine
Leucyl-alanine.	l-Leucyl-glycine.
Leucyl-leucine A.	l-Leucyl-d-alanine
Leucyl-leucine B.	l-Leucyl-l-leucine.
Leucyl-phenylalanine α .	d-Leucyl-l-leucine.
Leucyl-phenylalanine β .	d-Leucyl-d-leucine.
Leucyl-isoserine A.	l-Leucyl-d-leucine.
Leucyl-isoserine B.	d-Leucyl-l-asparagine.
Leucyl-asparagine.	l-Leucyl-l-asparagine.
Leucyl-aspartic acid.	l-Leucyl-d-tryptophane.
Leucyl-proline.	l-Leucyl-d-glutamic acid.
Phenylglycyl-glycine.	
Phenylglycyl-alanine A.	
Phenylglycyl-alanine B.	
Phenylglycyl-asparagine.	
Phenylalanyl-glycine.	l-Phenylalanyl-glycine.
Phenylalanyl-alanine.	
Phenylalanyl-leucine.	
Phenylalanyl-phenylalanine.	
Asparagyl-mono-glycine.	
Prolyl-alanine.	

Tripeptides.

Diglycyl-glycine (chloracetylchloride + glycyl-glycine ester).
 Alanyl-glycyl-glycine (α -bromopropionylbromide + glycyl-glycine ester).
 Leucyl-glycyl-glycine (α -bromisocapronylchloride + glycyl-glycine ester or + glycine anhydride + NaOH).
 Phenylalanyl-glycyl-glycine (phenyl- α -bromopropionylchloride + glycyl-glycine).
 Leucyl- α -leucyl-phenylalanine (α -bromisocapronylchloride + α -leucyl-phenylalanine).
 Leucyl-glycyl-phenylalanine (α -bromisocapronylchloride + glycyl-phenylalanine).
 Diglycyl-phenylalanine (chloracetylchloride + glycyl-phenylalanine).
 Diglycyl-cystine (chloracetylchloride + cystine).

stine (α -bromopropionylbromide + cystine).

stine (α -bromisocapronylchloride + cystine).

Aspargyl-dialanine (fumarylchloride + alanine ester).

Leucyl-alanyl-glycine A } (α -bromisocapronylchloride + alanyl-glycine).
 Leucyl-alanyl-glycine B }

Alanyl-leucyl-glycine (α -bromopropionylbromide + leucyl-glycine).

anine (chloracetylchloride + leucyl-alanine).

anine A }

Leucyl-alanyl-alanine B } (α -bromisocapronylchloride + alanyl-alanine).

Dialanyl-alanine (α -bromopropionylbromide + alanyl-alanine).

l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine ester).

l-Alanyl-glycyl-glycine (l-bromopropionylchloride + glycyl-glycine).

d-Alanyl-glycyl-l-tyrosine (d- α -bromopropionylchloride + glycyl-l-tyrosine).

l-Leucyl-glycyl-d-tryptophane (d- α -bromisocapronylchloride + glycyl-d-tryptophane).

Tetrapeptides.

Triglycyl-glycine (chloracetylchloride + diglycyl-glycine).

Dileucyl-glycyl-glycine (α -bromisocapronylchloride + leucyl-glycyl-glycine).

l-Leucyl-diglycyl-glycine (d- α -bromisocapronylchloride + diglycyl-glycine).

Pentapeptides.

Tetraglycyl-glycine (chloracetylchloride + triglycyl-glycine).

III. Synthesis of Polypeptides by Means of the Acid Chlorides of the Amino Acids and of the Polypeptides.

This, the simplest method of combining together two or more amino acids, is the one, in contradistinction to the previous one, by which the chain of amino acids can be lengthened at the carboxyl end of the molecule. It could not be employed at the commencement of Emil Fischer's researches, since the acid chlorides of the amino acids were unknown, and all attempts to prepare them had failed; but it is now of the greatest importance, as it admits of the preparation of any conceivable polypeptide, and it has also given us the knowledge of the most complex compound known by synthesis.

Although the acid chlorides of the amino acids themselves were unknown, it was found by Fischer that their carbethoxyl derivatives, as also those of the dipeptides which had been prepared, could be converted into their acid chlorides by the action of thionyl chloride, and that these compounds could be combined with the esters of the amino acids or of polypeptides, thus:—

Carbethoxyl-glycyl chloride and glycine ester yielded carbethoxyl-glycyl-glycine ester.

Carbethoxyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl diglycyl-glycine ester.

Carbethoxyl-glycyl-glycyl chloride and glycyl-glycine ester yielded carbethoxyl-triglycyl-glycine ester.

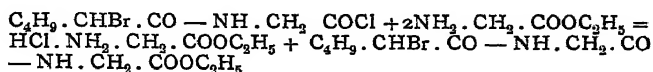
This last compound on hydrolysis gave the free acid, which contains four glycyl groups, and was the first known representative of the tetrapeptides.

In the same way derivatives of mixed polypeptides could be obtained, *e.g.*, carbethoxyl-glycyl-alanine ester. From it, by the action of ammonia, Fischer and Otto prepared the amide and, by saponification with soda, the free acid, but the preparation of the simple polypeptide could not be effected, since it was impossible to remove the carbethoxyl group without complete destruction of the molecule.

In 1904 Fischer found that the presence of a halogen acyl group in the molecule of an amino acid again allowed of the preparation of the acid chloride, *i.e.*, when the amino group of the amino acid was rendered stable, and that this compound was formed by the action of phosphorus pentachloride in the presence of acetyl chloride. As before, this acid chloride could be combined with the esters of amino acids or of polypeptides, *e.g.*—

Bromisocapronylglycine was converted into its acid chloride and

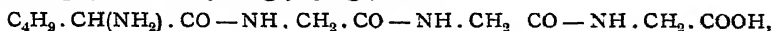
combined with glycine ester, when it yielded bromisocapronyl-glycyl-glycine ester,



which on subsequent saponification and treatment with ammonia, gave the tripeptide leucyl-glycyl-glycine,



If combined with glycyl-glycine ester and treated in the same way the tetrapeptide leucyl-diglycyl-glycine,

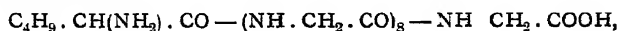


was obtained.

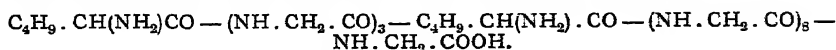
Not only was it possible to prepare the acid chloride of a halogen acyl derivative of an amino acid, but also that of a di-, tri-, etc., peptide by exactly the same means. Thus, the compound bromisocapronyl-diglycyl-glycyl chloride can be obtained, and by condensing it with the esters of amino acids and of polypeptides Fischer has prepared a hexa-, a hepta-, and a deca- peptide (see tabulation).

These compounds already exhibit the extraordinary possibilities of synthesis by this method. By continuing the process of preparing the acid chloride of a new polypeptide and again combining it with a polypeptide ester, the synthesis of the complex octadecapeptide, composed of fifteen glycine residues and three leucine residues, was effected in 1907. Its preparation is the best illustration of how this method lends itself to the synthesis of the polypeptides.

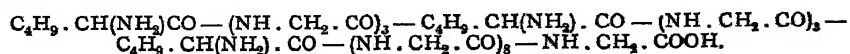
Bromisocapronyl diglycyl-glycine was converted into its acid chloride and combined with pentaglycyl-glycine. The resulting bromo compound was treated with liquid ammonia and the decapeptide l-leucyl-octaglycyl-glycine,



was obtained. This gave, on combination with bromisocapronyl diglycyl-glycyl chloride and subsequent treatment with ammonia, the tetradecapeptide, leucyl-triglycyl-leucyl-octaglycyl-glycine,



A repetition of the process of combining this new compound with bromisocapronyl-diglycyl-glycyl chloride and treating with ammonia yielded the octadecapeptide, leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine,



In the preparation of this octadecapeptide complete combination of the polypeptide with the acid chloride was very essential, since if the greater part of the compound be not used up, but remained unchanged, it was precipitated with the bromo compound on acidifying; this was only attained by using a very large excess of the acid chloride. At the same time there was the technical difficulty of frothing; this was overcome by shaking with glass beads in large flasks. Liquid ammonia was also necessary for the conversion of the halogen compound into its amino derivative. Analysis of the polypeptides hardly sufficed for the determination of their synthesis, since the variations in the figures are so small, but a determination of the bromine in the corresponding halogen derivative indicated that the synthesis was being effected in the stages represented.

This octadecapeptide has the highest molecular weight of any compound as yet prepared by synthesis and of which we know the constitution. Its molecular weight is 1213, a figure which far exceeds that of the fats, tristearin having a molecular weight of only 891. If the compound contained other amino acid residues, such as leucine, tyrosine, phenylalanine in the place of the glycine residues, the molecular weight would be increased two to three times.

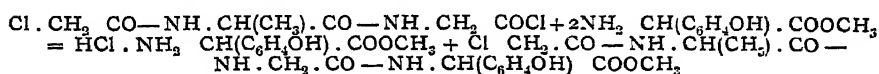
Such a figure of 3,000-5,000 has been found for the molecular weight of many proteins, and it would appear that they are composed of some twenty amino acids. The higher values of 12,000-15,000 which have been found for the molecular weight of other proteins, are, according to Fischer, very doubtful, since we have no indication of their purity, in spite of the crystallisability of many of them; the admixture of a small quantity of another protein might easily raise the value to this extent.

Fischer subsequently found that the acid chloride of other acyl derivatives of the amino acids could be prepared by the same process. Thus, by treating finely powdered hippuric acid with phosphorus pentachloride in the presence of acetyl chloride, he obtained hippuryl chloride, a compound which numerous investigators had tried to synthesise, but unsuccessfully. By combining hippuryl chloride with glycine ester, benzoyl-glycyl-glycine was obtained, and this compound, when converted into its acid chloride and combined with glycine, yielded benzoyl-diglycyl-glycine. In this way, by means of the acid chlorides, Fischer has prepared the same compounds which Curtius has prepared by means of hippurazide.

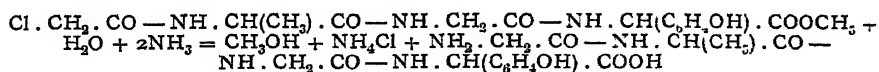
By applying this method of preparing the acid chlorides to the amino acids themselves, Fischer ultimately succeeded in obtaining the

amino acid chlorides, so that he was enabled to combine together any two amino acids in any order, without the necessity of preparing the corresponding halogen derivative. Polypeptides containing the natural optically active amino acids can thus be synthesised with ease, since the natural compound obtained by hydrolysis can be again used directly in the synthesis, and very often it is easier to prepare the natural compound than the synthetical one, which also requires separation into its stereoisomers.

The synthesis of polypeptides composed of different amino acids is most easily effected by this method. Those containing tyrosine are of particular interest, since the first natural tetrapeptide was isolated from silk in 1907, and was composed of glycine, alanine and tyrosine. Twelve isomers are possible for a tetrapeptide of this composition, but if the results of partial hydrolysis and subsequent anhydride formation be taken into account, this number is reduced to eight. Of these, glycyl-d-alanyl-glycyl-l-tyrosine was synthesised in 1908 by combining chloracetyl-d-alanyl-glycylchloride with l-tyrosine ester:—



saponifying the resulting chloro compound with caustic soda and treating with aqueous ammonia:—



This product, though it had many points of resemblance, such as precipitation by phosphotungstic acid, tannic acid, hydrolysis by trypsin, with the natural tetrapeptide, was not, however, identical with it; it differed mainly in its behaviour to ammonium sulphate, by which it was only salted out with great difficulty.

An attempt was made at the same time to prepare the isomeric d-alanyl-glycyl-glycyl-l-tyrosine; it failed on account of the difficulty of preparing pure α -bromopropionyl-glycyl-glycyl-chloride, but there seems no reason to suppose that Fischer will not overcome this small difficulty in preparing a desired compound, when he has overcome such vast difficulties already in connection with the synthesis of the polypeptides.

The accompanying table gives a list of the compounds prepared by this method.

POLYPEPTIDES SYNTHESISED BY METHOD III

Dipeptides.

Benzoylglycyl-glycine (hippuryl chloride + glycine ester).
 d-Alanyl-glycine (d-alanyl chloride + glycine ester).
 d-Alanyl-d-alanine (d-alanyl chloride + d-alanine ester).
 dl-Valyl-glycine.
 Valyl-alanine A.
 l-Leucyl-glycine (l-leucyl chloride + glycine ester).
 l-Leucyl-d-alanine (l-leucyl chloride + d-alanine ester).
 l-Leucyl-l-leucine (l-leucyl chloride + l-leucine ester).
 d-Tryptophyl-glycine (d-tryptophyl chloride + glycine ester).

Tripeptides.

Benzoyl-diglycyl-glycine (benzoyl-glycyl-glycyl chloride + glycine ester).
 Leucyl-glycyl-glycine (leucyl-glycyl chloride + glycine ester).
 Leucyl-glycyl-leucine (leucyl-glycyl chloride + leucine ester).
 Glycyl-l-asparagyl-l-leucine (chloracetyl-l-asparagyl chloride + l-leucine ester).

Tetrapeptides.

Leucyl-diglycyl-glycine (bromisocapronyl-glycyl-glycyl chloride + glycine ester).
 " " " (bromisocapronyl-glycyl chloride + glycyl-glycine ester).
 " " " (leucyl-diglycyl chloride + glycine ester).
 Glycyl-d-alanyl-glycyl-l-tyrosine (chloracetyl-d-alanyl-glycyl chloride + l-tyrosine ester).

Pentapeptide.

Leucyl-triglycyl-glycine (α -bromisocapronyl-diglycyl-glycyl chloride + glycine ester).

Hexapeptide.

Leucyl-tetraglycyl-glycine (α -bromisocapronyl-diglycyl-glycyl chloride + glycyl-glycine)

Heptapeptide.

Leucyl-pentaglycyl-glycine (α -bromisocapronyl-diglycyl-glycyl chloride + diglycyl-glycine).

Octapeptide.

Leucyl-hexaglycyl-glycine (α -bromisocapronyl-diglycyl-glycyl chloride + triglycyl-glycine).

Decapeptide.

Leucyl-octaglycyl-glycine (α -bromisocapronyl-diglycyl-glycyl chloride + pentaglycyl-glycine).

Dodecapeptide.

Leucyl-decaglycyl-glycine (bromisocapronyl-tetraglycyl-glycyl chloride + pentaglycyl-glycine).

Tetradecapeptide.

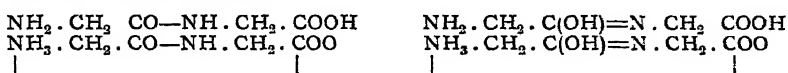
Leucyl-triglycyl-leucyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + l-leucyl-octaglycyl-glycine).

Leucyl Octadecapeptide.

Leucyl-triglycyl-leucyl-triglycyl-octaglycyl-glycine (bromisocapronyl-diglycyl-glycyl chloride + leucyl-triglycyl-leucyl-octaglycyl-glycine).

The Structure of the Polypeptides and Diketopiperazines.

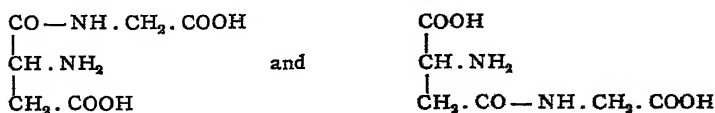
From the methods by which the polypeptides are obtained by synthesis it can only be concluded that their constituent amino acids are combined together in the form of acid amides; this method of combination also occurs in the case of the oxyamino acids, *e.g.*, leucyl-isoserine, where the ester method of combination was excluded by special investigations. The question of their structure, however, still remains very complex, if the controversy concerning the structure of the amides and amino acids, which has not yet been settled, be taken into account. There is the possibility of lactam and lactim forms and of the free amino acid and intramolecular salt; these are illustrated by the four formulæ for glycyl-glycine:—



For the sake of simplicity and since his observations have as yet led to no choice between the above formulæ, Fischer has adopted the first formula, but in certain of the polypeptides the observations suggest different structures, which will increase with the investigations upon the more complex polypeptides, thus leucyl-diglycyl-glycine in its amorphous state is easily soluble in alcohol, if the alcohol solution be warmed on the water bath, the crystalline tetrapeptide commences to separate out and in this state it is insoluble in alcohol.

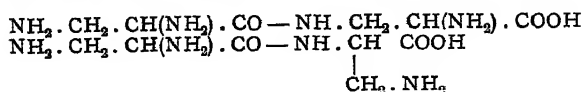
The carbethoxyl derivatives show another kind of isomerism; carbethoxyl glycyl-glycine ester, when saponified by alkali, yielded glycyl-glycyl carboxylic acid $\text{HOOC} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$; this compound on esterification by alcoholic hydrochloric acid yielded a neutral ester, and this compound was isomeric with the former one. The experiments of Leuchs (p. 18) have shown their exact nature, and they are designated as the α - and β -compounds. A similar isomerism occurs with carbethoxyl diglycyl-glycine ester and with the corresponding double amides.

Polypeptides, which contain amino dicarboxylic acids or diamino acids can also exist in isomeric forms; asparagyl-monoglycine can exist in the two forms,

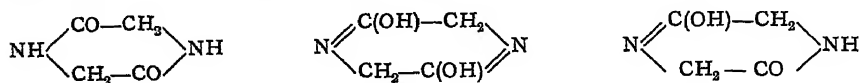


as also the dipeptide of diaminopropionic acid:—

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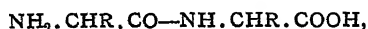
The diketopiperazines which are so closely related to the dipeptides can also occur in a keto- or -enol form; the possibilities are—



The existence of the -enol form was emphasised by the fact that in the hydrolysis of alanine anhydride by alkali, a transient formation of an alkali compound was observed.

The Configuration of the Polypeptides.

Excepting glycine all the amino acids employed in the previous syntheses contain an asymmetric carbon atom. According to the law of van't Hoff, the polypeptides will therefore occur in 2^n forms. Thus, a dipeptide,



containing two asymmetric carbon atoms will be capable of existence in the four active forms



of which the two first and the two last together will form a racemic compound.

A tripeptide can exist in 2^3 forms, *i.e.*, eight, a tetrapeptide in 2^4 or sixteen forms, etc.

The two inactive forms of a dipeptide are obtained when the two optically inactive compounds are coupled together by synthesis, and they appear first in the form of the corresponding halogen derivative,



A separation of the two racemic forms has been effected in certain cases at this stage, *e.g.*, leucyl-phenylalanine, but in the majority of cases only one product has been isolated. The formation of only one product in the reaction may be due either to the influence of stereoisomerism upon the combination of the compounds, which is especially noticeable when enzymes are concerned, or it may be due to a difference in the rate of combination of the two compounds, which was first observed by Markwald and Mackenzie with simple compounds. The latter explanation is the more probable, since when both compounds have been isolated, their amounts have been very different. There still remains the possibility that the single substance isolated is still a

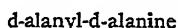
mixture of the two compounds, for the separation of mixed crystals of similar compounds is of the greatest difficulty.

Concerning the nomenclature of the two compounds where they have been isolated, the more insoluble is called the A compound and the more soluble the B compound. It has been possible to determine by the action of trypsin, which only hydrolyses the compound containing the naturally occurring amino acids, what combinations are present in them; thus, as alanyl-leucine A was hydrolysed by trypsin it must contain d-alanyl-l-leucine, and the two compounds must therefore be



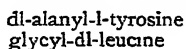
This has been proved by the later work upon the optically active polypeptides composed of these amino acids.

One product only can result when the two components consisting of the pure optically active amino acids are combined together, *e.g.*—

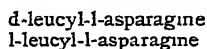


from d-alanylchloride and d-alanine ester.

Two products again result when one of the components is optically active and the other racemic. The various combinations of optically active tyrosine and aspartic acid with racemic leucine, alanine, etc., come into this category; they are designated as, *e.g.*—

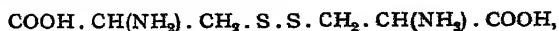


These compounds are not optical antipodes, and can therefore be separated by simple crystallisation. In the case of the leucyl-asparagines,



this separation has been effected, but in the majority of cases no separation was carried out, since the similarity of the isomers was so great that they formed apparent mixed crystals. Such a condition was termed by Fischer in 1894 "partial racemism". It occurs almost always when a racemic compound in combination with an active residue cannot be separated into its two isomeric forms by simple crystallisation.

Cystine, as its constitution shows,



resembles the tartaric acids in its stereochemistry; it is composed of two exactly similar halves, and it matters very little with which amino group combination is effected. But if it be combined with two mole-

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cules of a racemic acid chloride, *e.g.*, α -bromopropionyl chloride, three isomeric optically active compounds can result, namely:—

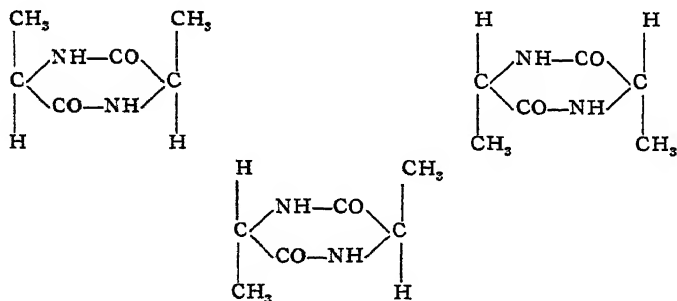
d-bromopropionyl-d-bromopropionyl-cystine
l-bromopropionyl-l-bromopropionyl-cystine
d-bromopropionyl-l-bromopropionyl-cystine

A yield of 71 per cent. of dibromopropionyl-cystine was obtained by Fischer and Suzuki and was apparently a definite substance. It was therefore regarded as the dl-compound, since its formation is independent of the formation of the dd- or ll-compounds which most probably would result in equal amounts.

The Configuration of the 2, 5- Diketopiperazines.

Diketopiperazines composed of two molecules of the same amino acids, *i.e.*, containing the same substituting group, can, according to theory, exist in four forms, which are comparable to those of the tartaric acids, namely:—

1. The active dextro form, containing the two dextro rotating molecules.
2. The active laevo form, containing the two laevo rotating molecules.
3. The inactive form, a mixture of 1 and 2.
4. The inactive form, containing a dextro and a laevo rotating molecule, as can be seen from the structural formulæ for the anhydrides of alanine:—



The substituting groups in the optically active forms are thus in the *cis*-position, in the inactive meso form in the *trans*-position.

The proof of the existence of these various forms was commenced in 1906 by Fischer and Raske in the case of the alanine anhydrides. They prepared the inactive *trans*-anhydride

1. by the action of ammonia upon l-alanyl-d-alanine ester ;
2. by the action of ammonia upon d-alanyl-l-alanine ester.

The active d-alanine anhydride had been previously synthesised in a similar way from d-alanyl-d-alanine ester, and an inactive anhydride

had been obtained by heating inactive alanine ester; this probably represents the inactive mixture of the dextro and laevo anhydrides; the other compound l-alanine anhydride has not yet been prepared.

The several forms of the diketopiperazines were synthesised in 1907 by Fischer and Koelker, who prepared

1. d-leucine anhydride from d-leucyl-d-leucine ester and ammonia ;
2. l-leucine anhydride from l-leucyl-l-leucine ester and ammonia ;
3. trans-leucine anhydride from d-leucyl-l-leucine ester and ammonia and from l-leucyl-d-leucine ester and ammonia.

Hydrolysis by alkali of these anhydrides should give the corresponding dipeptide, but in the case of the aminobutyryl-aminobutyric acid anhydrides Fischer and Raske have found that a steric rearrangement occurred; the dipeptide A was obtained both from the anhydride A and B; these had been prepared by the action of ammonia on the respective inactive esters, so that in this manner the dipeptide B can be converted into the dipeptide A.

The number of isomers of diketopiperazines containing two molecules of different amino acids is greater than when the two amino acids in the molecule are the same. It can be calculated from the number of asymmetric carbon atoms in the molecule just as in the open chain compounds; thus alanyl-leucine anhydride can exist in four optically active forms and two racemic forms:—

1. d-alanyl-d-leucine anhydride ;
2. d-alanyl-l-leucine „
3. l-alanyl-d-leucine „
4. l-alanyl-l-leucine „
5. a mixture of 1 and 4 ;
6. a mixture of 2 and 3.

The same diketopiperazines are obtainable either from the dipeptides alanyl-leucine or from the dipeptides leucyl-alanine, so that in fact the number of isomers of a diketopiperazine composed of two different amino acids is less than those of the isomers of the dipeptide composed of the same two amino acids.

At present only a racemic form prepared from leucyl-alanine by fusion has been obtained; its nature has not yet been determined.

The Properties of the Polypeptides.

The physical properties of the various polypeptides show generally much resemblance to one another, although many differences have been observed.

The majority are easily soluble in water; the exceptions amongst the dipeptides are, *e.g.*, dl-leucyl-glycine, leucyl-alanine, and leucyl-leucine; also phenylalanyl-glycine, phenylalanyl-phenylalanine, and some others; amongst the tripeptides, leucyl-alanyl-alanine A, phenylalanyl-glycyl-glycine, leucyl-glycyl-phenylalanine; amongst the tetrapeptides, dileucyl-glycyl-glycine. In contradistinction to the other polypeptides made up entirely of glycine units, the pentapeptide and the hexapeptide are soluble with difficulty even in hot water.

Of the complex polypeptides, the octapeptide, l-leucyl-hexaglycyl-glycine, is the most soluble in water, and the decapeptide, l-leucyl-octaglycyl-glycine, the least soluble; the solubility increases again in the case of the tetradeca- and octadecapeptides; their warm clear aqueous solutions become opalescent on cooling.

In general, the solubility in water of mixed polypeptides is greater than the solubility of the polypeptides made up of a single amino acid; the ready solubility of the dipeptides glycyl-l-tyrosine, leucyl-tyrosine, which contain the amino acids soluble with difficulty in water, should also be noted.

Most of the polypeptides are insoluble in alcohol; leucyl-proline is, however, an exception, for it dissolves both in alcohol and in acetic ester somewhat easily.

Those polypeptides which are soluble with difficulty in water, are dissolved easily by mineral acids and alkalies with the formation of salts; they are less soluble in acetic acid. In many cases they may be dissolved in alcohol if a few drops of ammonia be added; they separate out on boiling off the ammonia.

Certain polypeptides, for instance, leucyl-diglycyl-glycine, in the amorphous state are soluble in alcohol, but they are changed on warming into their insoluble crystalline form.

Most of the polypeptides melt above 200° C. and at the same time undergo decomposition. The dipeptides when fused are converted into their diketopiperazines. Certain of the glycine polypeptides are decomposed without melting. Leucyl-proline again, as in many other of its properties, is an exception, as it melts at 116-119° C.

The taste of the polypeptides is not sweet, like that of the amino acids, but slightly bitter; some of the isomeric polypeptides possess

distinct differences in their taste; thus leucyl-alanine is tasteless, but the two alanyl-leucines have a bitter taste. The presence of α -amino acids amongst polypeptides may even be recognised by their sweet taste, and the resemblance of the polypeptides in their bitter taste to the natural peptones is very remarkable.

The optically active polypeptides have generally a very high specific rotation in comparison with the amino acids; but the rotation is very changeable just as in other classes of compounds. Multirotation has not yet been observed. This property has proved very valuable in the study of the hydrolysis of the polypeptides by the action of enzymes.

The chemical properties of the polypeptides depend greatly on their complexity. Like amino acids, all the ordinary polypeptides, when their solutions are boiled with precipitated copper oxide, give blue, sometimes blue-violet solutions, and in this way differ from the diketopiperazines, whose solutions remain colourless, *i.e.*, they do not give copper salts. Leucyl-proline forms again an exception.

The high molecular polypeptides, such as the octa-, the deca- up to the tetradeca-peptides, give salts with mineral acids which are soluble with difficulty, but the lower ones give soluble salts as before mentioned.

The simple polypeptides, like the α -amino acids, give no precipitate with phosphotungstic acid, but this condition depends on the length of the polypeptide chain. Many tripeptides, such as leucyl-glycyl-glycine, give a precipitate with phosphotungstic acid in the presence of sulphuric acid if their solution be not too dilute, and this occurs with almost all the tetrapeptides. The derivatives of the diamino acids behave as expected in giving a precipitate with phosphotungstic acid.

The octa-, deca-, etc., peptides are immediately precipitated by phosphotungstic acid; they are also thrown down by tannic acid and by concentrated ammonium sulphate solutions. They resemble, in fact, many natural proteins and would have been regarded as such if they had been found in nature. They lack only the colour reactions due to the absence of tyrosine, tryptophane, etc., in their molecules.

✓ The biuret reaction is positive with the greater number of the polypeptides excluding the dipeptides. In the case of the glycine compounds it occurs first with the tetrapeptide, but it occurs with other tripeptides. It is distinctly intenser as the length of the polypeptide chain increases, and the colour is also more intense when the carboxyl group is esterified; this is especially noticeable in the case of triglycylglycine and its ester, the so-called biuret base. The same occurs when

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the carboxyl group is converted into the acid amide group; here one of the conditions necessary, as shown by Schiff, is added.

The polypeptides yield the same derivatives as the amino acids; the carboxyl group can be converted into the acid chloride and a halogen acyl group can be attached to the amino group. Further, the benzoyl, the carbethoxyl and the naphthalene-sulpho compounds can be easily obtained; the derivatives with the last mentioned are soluble with difficulty and may be made use of for characterising the polypeptides. The combination with phenylisocyanate also takes place readily, but these compounds are not so important as those of the amino acids for purposes of characterisation.

On the other hand, the esters of the polypeptides are of the greatest importance and they are prepared by the action of alcoholic hydrochloric acid. Hydrolysis of the polypeptide does not occur if prolonged heating be avoided, nor does hydrolysis occur when the esters are saponified by dilute cold caustic alkali. The esters have served in particular for the further synthesis of polypeptides and for the isolation of dipeptides from mixtures; on treatment with alcoholic ammonia, the dipeptide esters are converted into their diketopiperazines. They are not soluble in petroleum ether and they are soluble with difficulty in ether, and they thus differ from amino acid esters. Chloroform dissolves them, and in this solvent their combination with acid chlorides has been generally effected.

The polypeptides are attacked by nitrous acid with evolution of nitrogen, but the amino and imino groups show no great difference in their behaviour as might have been expected, and consequently a differentiation of the amino and imino groups in the protein molecule with nitrous acid seems impossible. They are not acted upon by potassium permanganate in the cold, but on boiling they are oxidised as was shown by Pollak in the case of glycyl-glycine.

The synthetical polypeptides are completely hydrolysed by boiling with concentrated hydrochloric acid for five hours; 10 per cent. hydrochloric acid at 100° C. hydrolyses them very slowly, and normal alkali has only a very slight action. Their hydrolysis by enzymes, especially by trypsin, is of such importance that a special section is required for the description of these results.

*The Action of Enzymes upon the Polypeptides.**I. The Action of Trypsin.*

One of the best proofs that the protein molecule is built up of amino acids coupled together by the methods devised by E. Fischer is given by the action of the various enzymes upon the synthetical polypeptides.

In 1903, soon after the synthesis of a few of the simple dipeptides and their derivatives had been effected, Fischer and Bergell investigated the action of an extract of pancreas upon them and they found that

glycyl-glycine	}	were not hydrolysed
β -naphthalenesulphoglycyl-d-alanine		
β -naphthalenesulpho-d-alanyl-glycine		
Di- β -naphthalenesulphotyrosyl-dl-leucine		
β -naphthalenesulphoglycyl-l-tyrosine	}	were hydrolysed
β -naphthaleneglycyl-dl-leucine		
Carbethoxyl-glycyl-dl-leucine		
Glycyl-l-tyrosine		
Leucyl-alanine		
Alanyl-leucine		
Leucyl-leucine		

from which it was obvious that several factors conditioned the hydrolysis by the enzymes of the pancreas, such as the nature of the dipeptide and its configuration: *eg*, the racemic compounds were hydrolysed asymmetrically, the natural component, such as l-leucine being split off from carbethoxyl-glycyl-dl-leucine, the remainder not being acted upon. The results coincide with the facts known with regard to the rapid separation of leucine and tyrosine from proteins by the action of trypsin, the other amino acids, such as glycine and alanine, are not obtained during the early stages of digestion.

Fischer and Abderhalden, in 1905, extended these observations by investigating the effect of pancreatic juice prepared by Pawlow from a pancreatic fistula and activated by enterokinase from duodenal juice, *i.e.*, by the action of pure trypsin upon a larger number of polypeptides, and they were able to divide the polypeptides into two distinct classes:—

Those Hydrolysed	Those not Hydrolysed
* Alanyl-glycine.	Glycyl-alanine.
* Alanyl-alanine.	Glycyl-glycine.
* Alanyl-leucine A.	Alanyl-leucine B.
* Leucyl-isoserine A.	Leucyl-alanine.
Glycyl-l-tyrosine.	Leucyl-glycine.
Leucyl-l-tyrosine.	Leucyl-leucine.
* Alanyl-glycyl-glycine.	Aminobutyryl-glycine.
* Leucyl-glycyl-glycine.	Aminobutyryl-aminobutyric acid A.

* These are racemic compounds.

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Those Hydrolysed.

- * Glycyl-leucyl-alanine.
- * Alanyl-leucyl-glycine.
- Dialanyl-cystine
- Dileucyl-cystine.
- Tetraglycyl-glycine.
- Triglycyl-glycine ester (Curtius' biuret base).

Those not Hydrolysed

- Aminobutyryl-aminobutyric acid B.
- Valyl-glycine
- Glycyl-phenylalanine.
- Leucyl-proline.
- Diglycyl-glycine.
- Triglycyl-glycine.
- Dileucyl-glycyl-glycine.

To which were added in 1907 the following optically active dipeptides :—

- d-alanyl-d-alanine.
- d-alanyl-l-leucine.
- l-leucyl-l-leucine.
- l-leucyl-d-glutamic acid.

- d-alanyl-l-alanine.
- l-alanyl-d-alanine
- l-leucyl-glycine.
- l-leucyl-d-leucine.
- d-leucyl-l-leucine.

The hydrolysis of these compounds by the enzyme was determined by the isolation of the individual substances. The isolation of the amino acids soluble with difficulty in water, namely, tyrosine and cystine, presented no great difficulty, since those compounds crystallised out during the process of hydrolysis, but in the other cases the amino acids required separation from unchanged dipeptide. The ester method here again proved its usefulness; the esters of the simple monoamino acids are easily volatile in vacuo and can be characterised by the methods previously described; those of the dipeptides are not volatile and are characterised by conversion into their diketopiperazines or anhydrides by the action of ammonia, which compounds are less soluble than the dipeptides themselves and are thus capable of separation by filtration.

By simply determining the change in rotation, especially when optically active polypeptides were investigated, an indication that hydrolysis was occurring was obtained; as soon as the rotatory power became constant it was assumed that complete hydrolysis had taken place and the solution was examined for the products of hydrolysis.

In all cases the activity of the ferment was first proved, and freedom from bacterial infection was specially guarded against and certified by control experiments.

An examination of the results of hydrolysis by trypsin shows that several factors have an important influence :—

1. The Structure of the Molecule.

Glycyl-alanine, $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CO} - \text{NH} \cdot \text{CH}(\text{CH}_3) \cdot \text{COOH}$, was not hydrolysed, but the isomeric alanyl-glycine, $\text{NH}_2 \cdot \text{CH}(\text{CH}_3) \cdot \text{CO} - \text{NH} \cdot \text{CH}_2 \cdot \text{COOH}$, was hydrolysed; again, alanyl-leucine A was hydrolysed, but not leucyl-alanine.

The order in which the amino acids are combined together in the molecule has therefore a very marked effect. Thus, when alanine is

* These are racemic compounds.

the acyl radical, as in alanyl-glycine, alanyl-alanine, alanyl-leucine, hydrolysis occurred, but the reverse happened when leucine, valine or aminobutyric acid were the acyl radicals; in the three cases, leucyl-alanine, leucyl-glycine and leucyl-proline, no hydrolysis took place; here the racemic compound was employed and the resistance might have been due to this factor, but the instance of l-leucyl-glycine appears to confirm the older result.

If tyrosine, isoserine and cystine stood at the end of the chain, trypsin hydrolysed the compound; in the only case examined where tyrosine, combined with β -naphthalene-sulphonic acid, acted as the acyl radical, there was no hydrolysis.

2. The Configuration of the Molecule.

This is of the greatest importance for a polypeptide to be hydrolysed by trypsin, as will be seen from the list of compounds published in 1907. Only those compounds containing the naturally occurring optically active variety of the amino acid are hydrolysed by trypsin.

The compounds marked with an asterisk are racemic and their hydrolysis was effected asymmetrically, only that portion of the molecule containing the natural stereoisomer being attacked. This explained the difference between alanyl-leucine A and alanyl-leucine B; the former probably consisted of d-alanyl-l-leucine + l-alanyl-d-leucine, the first of which contains the natural stereoisomers upon which the hydrolysis depends; the latter would consist of d-alanyl-d-leucine + l-alanyl-l-leucine. The later experiments of 1907 proved this supposition. It is more noticeable in the case of leucyl-leucine, which must have been l-leucyl-d-leucine + d-leucyl-l-leucine since l-leucyl-l-leucine is hydrolysed.

3. The Number of Amino Acids.

Only the various polypeptides containing several glycine radicals come at present under this heading. Several interesting details are at once apparent. Hydrolysis first took place when five glycine radicals, as in tetraglycyl-glycine, are combined together, although it occurred in the ester of triglycyl-glycine, or the biuret base of Curtius, which had been previously examined by Schwarzschild. The length of the glycine chain is therefore of importance, but an alteration in the carboxyl group may have an influence; it is worth noting that Warburg observed that leucine ester was hydrolysed by pancreatic juice, but whether this was due to the trypsin or the lipase in the juice has not yet been determined.

The fact that leucyl-glycyl-glycine was hydrolysed, but not the more complex dileucyl-glycyl-glycine, was probably due to the configuration of the dileucyl group.

4. The Nature of the Enzyme.

In the earlier experiments by Fischer and Bergell it was found that leucyl-alanine was hydrolysed by an extract of pancreas, it was not however hydrolysed by pure pancreatic juice. Such extracts probably contain other enzymes, more especially the autolytic enzyme, which produce the hydrolysis; the later work of Abderhalden and his co-workers upon the action of enzymes from various organs also show that polypeptides not hydrolysed by pure trypsin are attacked by these enzymes (see table, p. 48).

II. The Action of Pepsin.

Amino acids have been described by various authors as occurring together with the proteoses and peptones in a pepsin digest of proteins. One might have expected that pepsin would act upon certain of the synthetical compounds, especially those most easily hydrolysed by trypsin. Pure pepsin, prepared by Pawlow, had however no action upon glycyl-l-tyrosine, leucyl-alanine, leucyl-leucine, dialanyl-cystine, leucyl-glycine, and one must conclude that the chain of amino acids is not yet sufficiently long to allow of attack by pepsin. The amino acids obtained by the digestion of proteins probably arise by the action of other enzymes contained in the enzyme solution employed. Another explanation may be that pepsin acts upon other combinations of amino acids than those which are hydrolysed by trypsin.

III. The Action of Other Enzymes.

Not only are the synthetical polypeptides hydrolysed by the enzyme of the pancreas, but they are also hydrolysed by the enzymes occurring in the animal body.

It was found by Abderhalden and Bergell, in 1903, that glycyl-glycine when subcutaneously introduced into a rabbit was converted into glycine which appeared in the urine, whereas glycine if administered in a similar way was completely burnt up and was not excreted. Abderhalden and Rona subsequently showed that glycyl-l-tyrosine was burnt up in the organism of the dog when injected into the system, and Abderhalden and Samuely observed that this was also the case when cystine, dialanyl-cystine and dileucyl-cystine were subcutaneously introduced. Abderhalden continued these investigations with Teruuchi, with the result that the organism of the dog was found to be able to completely utilise glycyl-glycine, alanyl-alanine and diglycyl-glycine as well as the diketopiperazines, glycine anhydride and alanine anhydride, when they were given by the mouth, just as the animal can utilise proteins or amino acids, the nitrogen contained in these substances being eliminated as urea. To this series of polypeptides capable of being utilised by the

dog Abderhalden and Samuely added dl-leucine and racemic leucyl-leucine, and Abderhalden and Babkin added leucyl-glycine. These results differed from those of Wohlgemuth, who found that the rabbit excreted d-leucine when dl-leucine was given, but Abderhalden and Kautzsch have also found that the rabbit excretes d-leucine when somewhat large doses of dl-leucine are administered, whereas this animal can utilise dl-leucyl-glycine and dl-leucyl-glycyl-glycine. Abderhalden has since found that the rabbit excretes glycine, l-alanine, and d-serine when the diketopiperazines of these amino acids are administered, which points to their hydrolysis into the dipeptide before they are split into the amino acids.

The organs of various animals, such as the dog and rabbit, would thus appear to differ in their power of making use of synthetical polypeptides, but the animal organism as a whole is not so selective as the enzyme of the pancreas which hydrolyses the racemic dipeptide asymmetrically; in the body the racemic compound is completely burnt up, since no dipeptide composed of the optically active variety of the amino acid not occurring in a protein could be isolated. Further, the animal organism is able to utilise polypeptides not hydrolysed by pancreatic juice, so that if such polypeptides are present in the protein, they can still be utilised by the body although unaffected by trypsin.

Although these polypeptides are utilised by the organism of an animal and the nitrogen contained in them excreted as urea, it does not follow from the results of the experiments that these polypeptides are hydrolysed into their constituent amino acids previous to absorption, more especially those which are not acted upon by trypsin.

Great interest therefore is attached to the subsequent work of Abderhalden in conjunction with Teruuchi, Hunter and Rona, which was commenced in 1906 upon the action of extracts and of press-juices of various organs, prepared by Buchner's method of grinding up with sand, mixing with Kieselguhr and pressing out at a pressure of 100-300 atmospheres, whereby the cell enzymes are obtained. A large number of polypeptides were employed for determining the nature of these enzymes in the hope of finding differences between them, and dividing the proteoclastic enzymes into definite groups, especially as we regard enzymes as being extremely selective in their nature, and in the hope also of determining in which organ the hydrolysis of any particular polypeptide took place. An extract of pancreas was previously found to hydrolyse leucyl-alanine, which was not attacked by pure pancreatic juice, but the results show that the enzymes contained in the various organs are not so selective as pure trypsin in their action, and among themselves show decided differences as exemplified in the following tabulation:—

	Glycyl-glycine	di-Leucyl-glycine,	Dialanyl-cystine,	Glycyl-di-alanine	Glycine anhydride	Glycyl-L-tyrosine,	Leucyl-leucine,	Leucyl-phenylalanine	di-Alanyl-glycyl-glycine	di-Leucyl-glycyl-glycine,	di-Alanyl-glycine	Glycyl-di-leucine,	Diglycyl-glycine	Triglycyl-glycine
Pyloric Juice			o
Duodenal Juice	+
Yeast Juice (Endotryptase)		..	.			+
Papain	+
Juice of Nepenthes			o		
Juice of Germinating Wheat . . .	+	+	+
Juice of Germinating Lupine . . .	+	+	+
Juice of Mushroom . . .		+	.			o		+	..	+	
Ox, Liver Extract . . .	+	+	.			.	o	+	+	+
" " Juice . . .	+	+	...	+	o	.	o	+	+
" " Muscle Juice . . .	+	+	...	+
Rabbit, Liver Juice . . .	slight	slight		slight						
" " Kidney Juice . . .	+	+	...	+
" " Muscle Juice . . .	+	+	.	+	...	+
Dog, Muscle Juice . . .	+	+
" " Kidney Juice . . .	+
" " Liver Juice . . .	+	+
" " Intestinal Juice (Erepsin) . . .	+	+
" " Extract of Intestine . . .	+
Pig, Lenses from Eyes	o	..	+			+	.	+	.
Calf, Brain	o	..	o	o	+	.	+	.
Ox, Blood Serum	o	o
Rabbit, Blood Serum	+
Dog, Blood Serum	+
Horse, Mixed Blood Corpuscles	+	.	+	.	.	+	+	.	.
Horse, Red Corpuscles	+	.	..	+	.	+	..	+	.
Dog } Red Corpuscles	+	
Sheep } Red Corpuscles	+	
Rabbit } Red Corpuscles	+	
Horse, Blood Platelets	+
" " Leucocytes	o
" " Plasma . . .	o	..	.	o	o	o	+	o	+	o	+	+
" " Serum . . .	o	o	o	.	..	+	...	+	..	+	+
Ox, Plasma	+	..	o	+	.	+	..	+	..
" " Red Blood Corpuscles	+	..	+	+	...	+	..
" " Blood Platelets	o or slight	..	o	o or slight	.	+	+	.

Glycyl-L-tyrosine is readily hydrolysed by trypsin, but not by pepsin, and it therefore serves an excellent compound for determining whether a given proteolytic ferment behaves as a peptic or a tryptic enzyme. For this reason it was employed by Abderhalden and Rona to determine the nature of the enzymes contained in the pyloric and duodenal

juices. Since neither of these juices acted upon glycyl-l-tyrosine they must be regarded as peptic in their nature.

Abderhalden and Teruuchi used glycyl-l-tyrosine to determine the nature of the enzymes in yeast juice, *i.e.* endotryptase, in papain and in the juice of nepenthes. The two former hydrolysed it, and consequently they contain tryptic enzymes; the last had no action upon it, and the enzyme of nepenthes is therefore like pepsin in its action. These results confirm the observations of other investigators, and the confusion concerning the nature of these enzymes would appear to be now settled with certainty.

The proteoclastic enzymes occurring in the germinating seeds of wheat and the lupine appear, according to the results obtained by Abderhalden and Schittenhelm, to have a stronger hydrolytic action than trypsin, since they break up glycyl-glycine and dl-leucyl-glycine which are unaffected by the enzyme of the pancreas. The same holds good for the enzyme of the mushroom, although glycyl-l-tyrosine was not hydrolysed; this compound was apparently destroyed by other enzymes in the mushroom.

The enzymes of the various organs of the animal body have hydrolysed with few exceptions all the polypeptides upon which their effect has been studied. Leucyl-leucine was not hydrolysed by the enzymes of the liver of the ox, and in all probability this was due to the insolubility of the polypeptide. The other striking result is that glycyl-l-tyrosine was not hydrolysed by the enzymes of calf's brain, which attacked the other polypeptides upon which it acted. The only diketopiperazine so far investigated was glycine anhydride, and this was not converted into glycine; this result would point to the absence of anhydrides in the products absorbed from the intestine. In general, the enzymes of the organs are more powerful than trypsin and less selective in their action.

The most interesting and astonishing facts were obtained by the examination of the blood corpuscles, the plasma and serum. Red blood corpuscles and platelets of the horse (but not of the ox) hydrolysed glycyl-l-tyrosine, which was not attacked by white corpuscles obtained from lymph or from pus cells, nor by the plasma or serum. Plasma and serum both hydrolysed dl-alanyl-glycine, as also the tri- and tetrapeptides diglycyl-glycine and triglycyl-glycine, which proves that the enzymes in the plasma and serum are not trypsin (or erepsin) absorbed from the intestine. Red blood corpuscles hydrolysed diglycyl-glycine, and the splitting caused by the plasma and serum may be due to the presence of the enzymes of the red blood corpuscles, either excreted naturally, or produced during the separation of the constituents, which

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was not probably quite perfect, since it is well known that there are great difficulties to be surmounted in obtaining serum or plasma absolutely free from the red colour of the corpuscles.

On account of these results with serum Abderhalden and Rona investigated the action of human blood serum on glycyl-l-tyrosine in certain cases of disease, as also the urine. In some diseases no hydrolysis occurred, but in other diseases there was distinct hydrolysis. As yet no conclusions can be drawn from these results, as they require amplification both as regards the enzyme solution and the substrate. In no case had the urine any action upon glycyl-l-tyrosine; this seems at variance with the presence of an urotryptic enzyme which Cathcart studied in its action upon proteins.

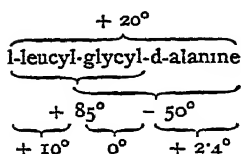
The hydrolysis of polypeptides by enzymes shows most conclusively that the protein molecule is built up of amino acids combined together in the form of acid amides, but there still remains the possibility that other modes of combination are present. In the animal body, proteins are acted upon firstly by pepsin, then by trypsin and then by the other enzymes. Pepsin does not hydrolyse any of the polypeptides, but it hydrolyses the proteins producing a mixture of some five or six proteoses and peptones. Trypsin hydrolyses the majority of the polypeptides, and it hydrolyses the proteins producing amino acids, together with a complex polypeptide, as Fischer and Abderhalden have shown, which contains all the proline and all the phenylalanine which are present in the protein. This complex polypeptide occurs in a modified form when a protein is hydrolysed first by pepsin and then by trypsin; some of the proline and the phenylalanine are obtained in the free state. The complex polypeptide is hydrolysed by the various intracellular enzymes in the organs of the body, since it is not excreted. These enzymes hydrolyse polypeptides which are not attacked by trypsin; such combinations are therefore probably contained in this complex. The enzymes in the various organs are extremely diverse in their nature: certain purine bases are acted upon by the enzymes of one organ but not by those of another organ, the arginase of the liver hydrolyses arginine (Kossel and Dakin), but not the similarly constituted creatine (as Dakin has recently shown). Enzymes are characterised by their highly selective nature; they act upon one definite substance or upon groups of substances, *e.g.*, the fats and the polypeptides. Pepsin does not act upon the polypeptides, but it hydrolyses the proteins; either the polypeptides are not sufficiently complex to be attacked by pepsin, or pepsin acts upon another mode of combination of the amino acids. One

mode of combination other than a polypeptide linking is present in arginine, and other modes are still possible. Bergell and Feigl have prepared combinations where two amino acids are combined to the ammonia by both their carboxyl groups, and these are not attacked by trypsin nor by pepsin. The oxyamino acids may be combined in the form of ethers with one another, or in the form of esters with other amino acids, and anhydrides of amino acids are possible. Further, diketopiperazine rings may occur, certain of which are easily hydrolysed by alkali.

Proteins, according to Cohnheim, are not hydrolysed by erepsin, the enzyme of the intestinal mucous membrane, which hydrolyses only the proteoses and peptones, converting them into amino acids; if proteins are previously hydrolysed by pepsin, they are then converted into amino acids by erepsin. Pepsin would therefore appear to have a special function rather than act like trypsin and the other enzymes, and it may attack one of the other possible linkings of amino acids. If it only produces some five or six products, there would only be the same number of such linkages.

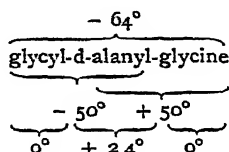
The optically active dipeptides, d-alanyl-d-alanine and d-alanyl-l-leucine, have been employed by Abderhalden and Koelker for comparing the activity of various enzymes, such as pancreatic trypsin, yeast endotryptase and intestinal erepsin, by observing the change in rotation they were able to determine the rate at which these polypeptides were hydrolysed, and they found that yeast endotryptase was the most active, erepsin attacked the dipeptide more slowly, and trypsin in forty-eight hours had scarcely hydrolysed it at all. Not only can the rate of change in rotation, which property was made use of for this purpose, be used to show differences in the various enzymes, but also it can be used for determining the rate of action (see monograph by W. M. Bayliss, F.R.S., on Enzyme Action) of the enzyme under various conditions as Abderhalden, in conjunction with Michaelis and Gigon, has shown.

By means of the change in rotation Abderhalden and Koelker have also attempted to determine at which point a tripeptide is first attacked by an enzyme. The specific rotation of l-leucyl-glycyl-d-alanine is $+20^\circ$, that of l-leucyl-glycine is $+85^\circ$, and that of glycyl-d-alanine is -50° . An increase in rotation would show that d-alanine was first separated and l-leucyl-glycine formed; a decrease in rotation would point to a separation of l-leucine and the formation of glycyl-d-alanine according to the following scheme:—



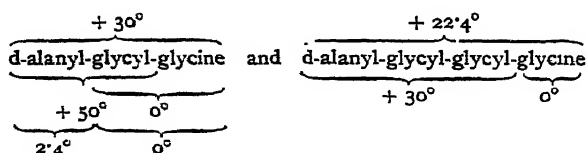
It was observed that the rotation at first increased to an extent of about 40 per cent., and under these conditions l-leucyl-glycine must be first formed and alanine separated off. Later, the rotation decreased which was due to the hydrolysis of l-leucyl-glycine. Glycyl-d-alanine was apparently not formed at all in the process of hydrolysis.

The tripeptide glycyl-d-alanyl-glycine was also investigated and the point of first attack determined. The rotations of the various compounds are :—



In the experiment the rotation decreased at first, was reversed in direction and then again decreased in amount. Glycine and d-alanyl-glycine must therefore have been formed first and the d-alanyl-glycine must then have been subsequently hydrolysed.

Further experiments were shortly afterwards made upon the compounds,



but here yeast endotryptase was also employed. By this enzyme hydrolysis was effected in such a way that the rotation in both cases gradually decreased which showed that d-alanine was first separated.

The action of trypsin upon d-alanyl-glycyl-glycine was also determined. The hydrolysis took place differently; the rotation decreased a little, increased considerably and then again decreased. This shows that glycine is first separated by trypsin, with the formation of d-alanyl-glycine, whereas endotryptase first split off d-alanine.

On account of this extraordinary difference the action of endotryptase upon l-leucyl-glycyl-alanine was examined. The rotation decreased and became negative, which showed that this tripeptide was completely

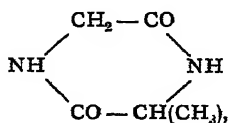
split open between the leucine and the glycine. With trypsin l-leucyl-glycine was first formed and d-alanine split off. We have thus a difference in the point of attack of enzymes of different origin, and may therefore possess a sharp means of differentiating between the various proteoclastic enzymes; it may prove of use in determining the nature of a complex polypeptide resulting by the hydrolysis of protein.

The Polypeptides Present in the Proteins.

The formation of a dipeptide by the hydrolysis of silk-fibroin was first described by Fischer and Bergell in 1902.

As is well known, silk-fibroin readily dissolves in cold concentrated hydrochloric acid ; if alcohol be then added, a product, called sericoïn by Weyl, is precipitated, but if the silk-fibroin be allowed to stand in contact with three times its quantity of concentrated acid for about twenty-four hours, alcohol no longer produces such a precipitate, and the solution contains the hydrochloride of a peptone. On concentration in vacuo, when freed from hydrochloric acid, a mass was obtained which had a bitter taste, was very soluble in water and gave strong biuret and Millon reactions, and which was very like peptone in its properties. When dissolved in water and digested in ammoniacal solution with trypsin, this peptone lost the whole of the tyrosine which it contained, and was converted into another peptone composed of 40·1 per cent. glycine and 28·5 per cent. alanine. From this compound, when heated with baryta water, ammonia was evolved, and the solution, freed from baryta, on evaporation yielded crystals; these were treated with β -naphthalene-sulphochloride, and a compound was obtained which was apparently β -naphthalene-sulpho-glycyl-alanine, though it could not be absolutely identified with the synthetical product of this composition.

The further attempts to again prepare this substance did not succeed, since the exact conditions leading to its formation could not be repeated, but in 1906 Fischer and Abderhalden obtained the anhydride of this body by a new method which they had discovered for isolating such compounds when mixed with amino acids and higher polypeptides. This method depends upon the different behaviour of the esters of these compounds; those of the simple mono-amino acids are easily volatile in vacuo and are therefore easily removed, whereas those of the dipeptides are converted by the action of ammonia into their anhydrides or diketopiperazines which crystallise readily and are therefore easily separated from the esters of the higher polypeptides. They thus obtained a methyl diketopiperazine,



which was identical with a synthetical product prepared from glycine and d-alanine, and which yielded glycine and d-alanine on hydrolysis.

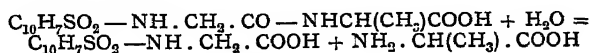
It resulted by the hydrolysis with 70 per cent. sulphuric acid followed by trypsin, and by the hydrolysis with hydrochloric acid.

This diketopiperazine could arise both from glycyl-d-alanine and d-alanyl-glycine by the above method. It could also arise by synthesis from glycine and d-alanine under the conditions of the experiment; control experiments were carried out to determine this possibility, and they showed that this was impossible so that there was no doubt concerning the presence of a dipeptide amongst the products of hydrolysis. Since this dipeptide was resistant to trypsin it was most probably glycyl-d-alanine and not d-alanyl-glycine which is easily hydrolysed by this enzyme.

At the same time another diketopiperazine, glycyl-l-tyrosine anhydride, was also obtained; its nature was established a little later by identification with synthetical glycyl-l-tyrosine anhydride prepared from the ester of chloracetyl tyrosine and ammonia. In one experiment its yield amounted to 4.2 per cent. of the silk-fibroin employed.

In the same way by the hydrolysis of elastin with 70 per cent. sulphuric acid and by the action of ammonia upon the esters, a product was isolated which was composed of glycine and l-leucine and which was identical with synthetical glycyl-l-leucine anhydride.

In 1907 Fischer and Abderhalden definitely showed that the first diketopiperazine isolated from the hydrolysis products of silk-fibroin was derived from glycyl-d-alanine. Silk-fibroin was partially hydrolysed by hydrochloric acid and then precipitated by phosphotungstic acid. A portion of the filtrate from this precipitate, after removal of the excess of phosphotungstic acid, was treated in alkaline solution with β -naphthalene-sulphochloride and a product was obtained which was identical with β -naphthalene-sulpho-glycyl-d-alanine, further proof of this was given by its careful hydrolysis with dilute hydrochloric acid when the dipeptide chain was split, but the naphthalene-sulpho radical not removed; β -naphthalene-sulpho-glycine and d-alanine were obtained according to the equation.—



From the remainder of the filtrate, glycyl-d-alanine anhydride and a small quantity of glycyl-l-tyrosine anhydride were obtained by the action of ammonia upon the esters, as well as another product which was most probably d-alanyl-l-serine anhydride.

An examination of the phosphotungstic acid precipitate showed that it contained several products of a complex nature. From them a substance was isolated which consisted of two molecules glycine, one

molecule alanine, and one molecule tyrosine, *i.e.*, a tetrapeptide. It had a molecular weight determined by the freezing-point method of about 350, was easily soluble in water, insoluble in alcohol, and was precipitated from its solution in flakes by saturation with ammonium sulphate or sodium chloride, as also by nitric or acetic acid. The synthetical pentapeptide l-leucyl-triglycyl-l-tyrosine behaves in a similar way so that great complexity, as formerly believed, is not an essential condition for precipitation by ammonium sulphate. By the action of trypsin tyrosine was split off, and on partial hydrolysis glycyl-d-alanine anhydride and glycyl-l-tyrosine anhydride were obtained.

In the same year (1907) the products obtained from elastin by partial hydrolysis were shown by Fischer and Abderhalden by the same methods to contain—

1. d-Alanyl-l-leucine. The anhydride of this dipeptide was also obtained; it probably arose from this dipeptide, but it can also be formed from the isomeric l-leucyl-d-alanine, whose presence amongst the products is not excluded.

2. Alanyl-proline anhydride, from which d-alanine and proline were obtained on hydrolysis.

3. Glycyl-valine anhydride, which was identical in its properties, except the melting-point, with the synthetical compound.

From gliadin Fischer and Abderhalden have also isolated a dipeptide by these methods, namely, l-leucyl-d-glutamic acid, which they identified with the synthetical substance, Abderhalden and Funk have isolated leucinimide, l-phenylalanyl-d-alanine anhydride by acid hydrolysis from casein, and probably also leucyl-valine anhydride.

In addition to these dipeptides isolated by Fischer and Abderhalden, Osborne and Clapp have obtained a crystalline substance by the acid hydrolysis of gliadin, which yielded proline and phenylalanine on further hydrolysis, and Levene and Beatty have isolated a dipeptide composed of glycine and proline from the products resulting by a trypsin digestion of gelatin. The exact nature of these bodies has still to be determined by comparison and identification with the synthetical substances, and not until this has been done can their presence in the molecule be accepted with certainty.

The appended list gives the polypeptides which have so far been isolated from proteins and therefore of the combinations of amino acids which occur in the protein molecule:—

Glycyl-d-alanine anhydride in silk-fibroin.

Glycyl-d-alanine in silk-fibroin.

Glycyl-l-tyrosine anhydride in silk-fibroin.

d-Alanyl-l-serine anhydride (?) in silk-fibroin.
Glycyl-l-leucine anhydride in elastin.
d-Alanyl-l-leucine anhydride in elastin.
d-Alanyl-l-leucine in elastin.
d-Alanyl-proline anhydride in elastin.
Glycyl-valine anhydride in elastin.
Leucinimide in caseinogen.
l-Phenylalanyl-d-alanine anhydride in caseinogen
l-Leucyl-d-valine anhydride in caseinogen.
l-Leucyl-d-glutamic acid in gliadin.
Dipeptide (proline + phenylalanine) in gliadin.
Dipeptide (proline + glycine) in gelatin.
Tetrapeptide (2 glycine + 1 alanine + 1 tyrosine) in silk-fibroin

It is only by the knowledge of the properties of the synthetical compounds that Fischer has been able to invent methods for isolating them from the mixtures which result by the hydrolysis of the proteins and to identify these compounds. The extension of the study of the higher polypeptides, more especially of the mixed polypeptides, will lead without doubt to the isolation of greater complexes from the products of partial hydrolysis of the proteins, such as the separation of the proteoses and peptones, which from the results so far obtained appear to be more simple than was previously supposed, if salting out by ammonium sulphate of polypeptides containing four and six units, of which tyrosine is one, be taken as a typical example.

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